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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered) 20030116010

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER RCS-MEDDH-288 (R1)	2. GOVT ACCESSION NO. AD-A154263	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Annual Progress Report - Fiscal Year 1982		5. TYPE OF REPORT & PERIOD COVERED Annual Report 1 October 1981 30 September 1982
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Author Index - page xvii		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Frederick, Maryland 21701-5011		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 3M161102BS10 3M162770A871 3M162770A870 3A161101A91C
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Research and Development Command Fort Detrick, Frederick, Maryland 21701-5012		12. REPORT DATE 1 October 1982 13. NUMBER OF PAGES 201
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) UNCLASSIFIED 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)
Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Human volunteers,	Bacterial Diseases,	Therapy,	Vulnerability,
Biological Warfare,	Rickettsial Diseases,	Pathology,	Host.
Viral Diseases,	Laboratory Animals,	Defense,	
Prophylaxis,	Parasite,	Metabolism,	
Identification,	Biochemistry	Malaria,	

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A report on the research program of the U. S. Army Medical Research Institute of Infectious Diseases on Medical Defense Against Biological Agents (U) for Fiscal Year 1982 is presented.

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EDITOR'S NOTE

This FY 1982 Annual Progress report is a general review of research activities of the U. S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, conducted on Medical Defense Against Biological Warfare Agents (U) under Projects 3M161102BS10, 3M161102BS10, and 3M162770A870 and In-House Laboratory Independent Research, Project 3A161101A91C.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

TITLE ON ITEM CORRECT

Cont'd

FOREWORD

I. USAMRIID's MISSION

The formal mission tasking USAMRIID reads as follows:

Perform studies on the pathogenesis, diagnosis, prophylaxis, treatment, and epidemiology of naturally occurring infectious diseases of military importance with emphasis on problems associated with the medical defense against biological agents and on those microorganisms which require special containment facilities. Originator supplied keywords include:

By DOD directive and further Army guidance, USAMRIID performs its Biological Agent Medical Defense research in support of the needs of the three services. This mission, and all work done at USAMRIID, is in keeping with the spirit and letter of both of President Nixon's 1969 and 1970 Executive Orders renouncing the use of biological and toxin weapons, and the U.N. Convention (Against) . . . Bacteriological (Biological) and Toxin Weapons . . . of 1972.

II. DISSEMINATION OF INFORMATION:

All work conducted at USAMRIID is unclassified. Results are published in peer-reviewed scientific literature, when accepted, as well as in annual reports. Oral and poster session presentations are given at meetings of numerous national and international scientific societies. Results of value to organizations outside the U. S. Department of Defense are shared willingly, in the hope that such sharing will result in additional information on the validity of scientific results or the efficacy of new products, such as vaccines, other biologicals, or drugs. Numerous intra-U. S. and international collaborations exist and are encouraged to expand. USAMRIID established an Office of Technology Transfer during FY 82 in order to fully implement the Stevenson-Wydler Technology Innovation Act of 1980. USAMRIID prints a cumulative bibliography of published articles, which may be obtained by a request to the Editor, USAMRIID, Fort Detrick, Maryland 21701.

III. THE STRATEGY OF THE PROGRAM:

A. The program rests on the judgment that both natural infectious diseases and potential biological warfare threats exist which could seriously interfere with the functions of U.S. forces. The first requirement for constructing the USAMRIID program is to arrive at an assessment as to which microbial and toxin agents are the highest priority threats. Those agents for which existing medical defenses are adequate are set aside. Those agents being addressed by other agencies within the U.S. or elsewhere are likewise set aside. From the refined list the available resources are applied in priority derived from considerations of the severity of their threat and the scientific feasibility of developing improved medical defenses against the agent.

B. The microorganisms or toxins being addressed (* = highest priority) or used in comparative studies during the period of this report were:

Bacterial Organisms

*B. anthracis** and other *Bacillus* species
F. tularensis
L. pneumophilia

S. pneumoniae
S. typhimurium
P. multocida
B. bronchoseptica
V. parahemolytica
S. typhi
S. somnei

Viral Organisms

Lassa fever virus*
Ebola fever virus*
Korean hemorrhagic fever virus* (Hantaan)
Rift Valley fever virus*
Bolivian hemorrhagic fever virus (Machupo)
Argentinian hemorrhagic fever virus* (Junin)
Dengue fever virus
Congo/Crimean hemorrhagic fever virus*
Sandfly fever virus
Eastern equine encephalitis virus
Venezuelan equine encephalomyelitis virus
Japanese encephalitis virus
Chikungunya virus
Pichinde virus
Yellow fever virus
Keystone virus
Influenza virus
Punto Toro virus
Vesicular stomatitis virus
Lymphocytic choriomeningitis virus
Mozambique virus
Marburg virus
Semliki forest virus
West Nile virus
Banzi virus

Rickettsial Organisms

C. burnetii

Parasitic Organisms

P. falciparum
P. inui

Toxins

Diptheria
Botulinum A-G*, including C2
Anthrax toxins*
T-2 and related tricothecene mycotoxins*
Saxitoxin*
Tetrodotoxin
Staphylococcal enterotoxin
Beta-bungarotoxin

IV. Goals

For each of the agents being addressed, the goals are:

A. Pathogenesis: Sufficient knowledge must be gathered concerning the biology of the agent and the responses of the infected or intoxicated host (generally in animal models) to provide a basis for progress in the applied goals listed below. Useful cell cultures, organ cultures, and a variety of laboratory animal models must be developed and exploited for the insight they can provide on the pathogenic processes in man, because information is limited for many of the human diseases of concern. Epidemiologic studies are conducted to gain insights into the transmissibility of these diseases.

B. Improved Diagnosis: Because the choice of medical interventions for either the prevention or the treatment of infectious/toxic disease can only be optimized when the precise infecting/intoxicating agent is known, the ability to make a rapid and specific identification of the causative agent is an important component of a system for medical defense. Ideally, there should be the capability to confirm the identity of organisms isolated from the environment, or to detect the antigen in appropriate clinical samples taken early in the course of disease. It is also important to detect antibodies from clinical patients in late illness or convalescence. The technology requirements should be suitable for use throughout the military medical system, including field facilities operating with austere resources. Not only must the threat agents of major concern be identified, but those more common agents which must be considered in a full differential diagnosis must also be identified with equal specificity.

C. Prevention: Immunization is the most effective, convenient, and economical means to reduce the impact from infectious disease on military forces. This goal, then, commands nearly half of USAMRIID's resources. The bulk of this effort is devoted to preliminary or feasibility studies in laboratory animals. Only a few vaccines are in the immediate preclinical stages of development. A sizeable number of FDA-approved Investigational vaccines are being used to protect laboratory personnel. Vaccine development is expected to continue as a major USAMRIID theme, since technological advances often allow the improvement of vaccines which were once state-of-the-art accomplishments. The application of modern biology to vaccine development is presenting opportunities and challenges not foreseen a few years earlier. Passive immunization, active immunization using killed or living attenuated whole agent, or immunization with sub-unit antigens achieved by older or newer molecular methods are options which must be evaluated comparatively for each disease, to arrive at the optimum immunizing method for military forces in various scenarios.

D. Treatment: An unexpected disease outbreak can preempt the opportunity to use prevention. Treatment then becomes the major medical means to limit damage to the individual and to maintain military force effectiveness. For many of the diseases of concern to USAMRIID, specific treatments which will reverse pathology have not yet been developed. Therefore, treatment strategy must consist of optimal supportive care to give the host defenses sufficient time to respond and eliminate the disease or toxemic insult. For these reasons, research on improving treatments at USAMRIID has emphasis on developing new specific treatments and on maximizing the effectiveness of supportive care of the infected or toxemic patient.

V. SUMMARY OF TRENDS IN FY 82

A. Since many programs at USAMRIID are multidisciplinary and carried out by multiple investigators in shifting consortia over several years, the material covered by this annual report may give only a fragmentary insight of the overall program and its progress. In the following sections, trends and accomplishments are highlighted.

B. General Progress Highlights during FY 82: FY 1982 was a highly productive and eventful year for USAMRIID, with its research programs producing much new data. The success of the overall USAMRIID program during FY 82 is indicated by the selection of the Institute as the 1982 Winner of the Army "Laboratory of the Year Award" and the First Place Award in the 1982 Army "In-House Laboratory Independent Research" competition. USAMRIID had previously won each of these awards in 1978.

The major realignments and shifts of program emphasis described in FY 81 (in response to USAMRDC guidance, SPEC/SPF guidelines and emerging information on BW activity in Southeast Asia) were fully implemented in FY 82. Much research progress emerged from the new studies on anthrax and the low molecular weight toxins. The study of field samples collected in Africa, Asia and South America became an effective seroepidemiology program. New diagnostic methods for numerous microorganisms and toxins were devised using ELISA methodology and the production of new diagnostic reagents included monoclonal antibodies. However, a consolidated and unified procedure for all field laboratory diagnostic attempts has not as yet been defined.

C. Research areas given added emphasis during FY 82:

The program initiated to study tricothecene, marine, and other low molecular weight toxins was expanded to its full operational capacity, and was supported by a new and integrated research contract program.

The anthrax program also achieved broad operational function, focused on exploiting the FY 81 discovery at USAMRIID of toxin-controlling plasmids, and the introduction of recombinant DNA technologies for the first cloning of the protective antigen gene.

The basic virology program was expanded. It reached the functional capacity to do oligonucleotide mapping and fingerprinting of key arenaviruses, bunyaviruses and closely related species.

The antiviral drug program increased its capabilities for screening to identify promising new compounds and for defining their unique mechanisms of antiviral activity.

The diagnostic program continued to expand with the production of new monoclonal and polyclonal antibodies, purified antigens, analytic technologies, and the comparative testing of readout instruments.

The epidemiology effort expanded overall, with added emphasis being placed on identifying, a) the geographical extent of antibodies to Hantaan-like viruses in rodent species, b) the possible insect and mammalian reservoirs of important African viruses, and c) the over-wintering mechanisms of insect-borne viruses.

Additional emphasis was also placed to determine why some inactivated vaccines may not afford protection against organisms delivered via an aerosol in contrast to the protection provided by attenuated vaccines.

D. Research Areas completed or given reduced efforts in FY 82:

USAMRIID work on the Legionella and Legionella-like bacteria is virtually complete. Planned work on the molecular characterization of Staphylococcal enterotoxin C and other staphylococcal exoproteins has been completed, as has work on the diphtheria and pseudomonas exotoxins. Some aspects of the in-house antiviral drug screening program are being transferred to research contracts.

E. List of significant accomplishments for FY 82:

1. USAMRIID was selected as winner of the Laboratory of The Year Award in competition with all other U.S. Army laboratories.

2. USAMRIID was selected as the top Army laboratory in the ILIR competition for the FY 82 Award.

3. A total of 49 research papers was published in 1982.

4. A newly renovated area was opened to house the Animal Resources Division and its normal laboratory animals.

5. A new teaching Course entitled "Medical Defense Against BW and Highly Communicable and Infectious Agents" was developed. This was presented a) for senior tri-service physicians specialized in infectious diseases and preventive medicine; b) for senior medical personnel involved in Rapid Deployment Force planning; c) and for Air Force residents in Preventive Medicine. This Course has now been listed in Cir 40-82-5 as a formal PPSCP Courses to be held in future years.

6. The NRC/NAS Postdoctoral Research Fellowship Program continued to expand at USAMRIID, with 10 Fellows participating in FY 82.

7. The expanded research program on anthrax included researchers from five different divisions. The edema factor (EF) shown in FY 81 to be an adenylate cyclase was further characterized and found to resemble mammallian cyclase quite closely. Additional studies were done on the anthrax plasmid (discovered at USAMRIID in FY 81) to determine how plasmids from various strains controlled exotoxin production. The protective antigen (PA) coding gene was localized in the plasmid and, using recombinant DNA technology, was successfully cloned into an E. coli. Non-PA components of the Anthrax bacillus were identified and tested for immunogenicity. New diagnostic and cultural measures were devised and tested. Phagocytic and bactericidal mechanisms were subjected to initial studies at the molecular level.

8. The expanded trichothecene research program was highly productive, completing studies on laboratory safety, decontamination procedures, a variety of detection methods, quantitative toxicity and pathophysiology in several animal models. In vivo toxin distribution, mechanisms of action, prophylaxis and therapy studies, and aerosol susceptibility studies also began. Initial studies emphasized T-2, but related mycotoxins are now being incorporated into the program. Considerable data concerning mechanisms of T-2 action were produced using cultured cells.

9. Studies initiated on the low molecular weight marine toxins included laboratory safety, detection methods, whole animal toxicity, and therapy.

10. Progress was made in toxoiding methodologies and in the molecular characterization of botulinum neurotoxins. Type E toxoid was tested in volunteers. Nerve cell cultures and synaptosomes were used for studying the molecular aspects of neurotoxin action.

11. In collaboration with contractors, fractionation methods were used to create a supply of human pentavalent botulinum IgG for therapeutic use, and a pool of despeciated septavalent equine origin $F(ab)_2$ antibodies, also for therapeutic use.

12. New neurophysiology laboratory studies demonstrated differences among the botulinum neurotoxins and discovered some synergism between experimental drugs used to restore nerve transmission.

13. The primary amino acid sequence of Staphylococcal enterotoxin C1 was defined.

14. An additional 78 potential antiviral drugs were evaluated in an *in vitro* screening program against Rift Valley fever, VEE, Pichinde, sandfly fever, vesicular stomatitis, and yellow fever viruses.

15. Didemnins (compounds isolated from marine tunicates) were introduced into the *in vitro* antiviral drug screening program and shown effective against certain militarily important viruses.

16. The reversible hematological toxicity in monkeys of an antiviral drug, ribavirin, was shown to involve both diminished red cell production and increased red cell destruction.

17. Entomology program studies have attempted to elucidate the vector potential and taxonomy of suspected arthropod vectors of Rift Valley fever virus. Several Egyptian mosquito species were shown to transmit the virus, but none could pass it transovarially. Species of sandflies or midges were not found to be candidates for field transmission. Field studies were initiated to extend these studies of RVF virus transmission in Kenya, and of EEE virus transmission in the Pocomoke swamp in Virginia.

18. Pathogenesis of Junin virus infection of guinea pigs was studied after aerosol exposure. Inbred guinea pigs were also used to study the role of T-cells, complement, antibodies, immunosuppression and antiviral drugs on the pathogenesis of disease.

19. Pichinde virus infections in Strain-13 guinea pigs were used as a model for studying hemorrhagic viral diseases, with emphasis on coagulation system changes, cardiovascular decompensation, and other mechanisms of disease progression.

20. Additional studies were done to characterize the disease process, immune response, epidemiologic spread, and virus culture methods for Ebola virus.

21. Lassa virus strains and antibody responses were characterized in great detail. The slow-forming neutralizing antibody has protective efficacy, especially against geographically similar strains, and can be concentrated effectively to increase the titer. A naturally attenuated Mozambique virus provides cross protection in animal models and thus becomes a condidate for a live virus vaccine.

22. Oligonucleotide mapping was used as a technique for estimating homology between dengue virus strains and for the preliminary characterization of Junin virus RNA.

23. Multiple purified samples of M segment RNA were prepared from RVF virus and sent to a contractor for gene cloning. A DNA clone has been isolated which appears to code for a portion of one RVF virus coat protein; this initial success suggests that it will be possible to produce a cloned sub-segment RVF vaccine.

24. The internalization of certain viruses into cells was shown to require initial binding to cell receptors. Virus was then transported sequentially into nonlysosomal and lysosomal vesicles. Lysosomal enzymes eventually functioned in viral uncoating before viral replication could begin.

25. The causative virus (Hantaan) of Korean hemorrhagic fever was purified, its electron microscopic appearance was characterized, and the virions were shown to contain three separate and unique RNA species and at least 2 resolvable proteins. A plaque assay was standardized for this virus.

26. Rat serum and tissues from many parts of the world were demonstrated to have antibody titers against Hantaan virus.

27. The production and comparison of various RVF virus strains and other viral antigens and antibodies was initiated for use with diagnostic ELISA methods. Testing time was reduced from 3 days to 1.

28. The use of ^{60}Cobalt irradiation was investigated for inactivation of viruses to be used as antigens in rapid diagnostic tests.

29. In continued studies, the chemiluminescent response of human neutrophiles was used as a relatively sensitive bioassay for identifying certain bacteria by a rapid technique for laboratory (rather than field) use. Bacterial chemiluminescent responses proved relatively nonsensitive for identifying trichothecene toxicity.

30. Additional studies were conducted in man on the immunological response to killed RVF vaccine. Serum samples and virus strains from different geographic areas were tested and interrelated.

31. Seed stocks of chikungunya virus were developed and tested to obtain a genetically stable new strain with growth characteristics suitable for vaccine production.

32. A search was initiated for new rodent models to improve the standardization of Q fever vaccines. Inbred mouse lines are receiving further study. Studies were continued on the Phase I antigen of C. burnetii to separate the protective components from those which cause adverse vaccine reactions. The amino acid, sugar, and lipid components were quantified.

33. The effectiveness of living-attenuated vaccines versus killed vaccine preparations for protecting animals against aerosol challenge was studied for VEE and anthrax.

34. Aerosol risk-assessment studies failed to define differences in the guinea pig model which could explain the pathogenesis of "pneumonic" versus "Pontiac-fever" forms of Legionella infections in man.

35. The toxin produced by Legionella and Legionella-like bacteria was studied for its effects on phagocyte function.

36. Studies were conducted in several different animal model systems to evaluate the interrelationships between physical exercise and infectious illness. In general, physical conditioning prior to infection appeared to improve host resistance, while acute infections consistently impaired physical performance capabilities. Bacterial and viral infection caused different responses on skeletal and cardiac muscle functions and chemistry. The effects of exhaustive exercise on the infectious process also differed depending on the etiology of the infection.

37. Endocrine responses and hormone-induced changes during infection focused upon growth hormone, prolactin, adrenal glucocorticoids and especially the role of insulin and its cell-surface receptors.

38. A jacket-and-tether system, permitting long-term intravenous infusions in monkeys, was used further for testing various combinations of intravenous nutrients with altered energy and amino acid content for their efficacy in treating model infections.

39. Studies were continued in volunteers to test new antimalarial drugs against resistant species, and in other volunteers to gather further data on FDA-approved investigational vaccines.

VI. EXTRAMURAL RESEARCH:

While this report deals principally with USAMRIID's in-house effort, total program progress is the result of the combination of the in-house effort augmented and supplemented by efforts by contractors from academia and industry. Individual contractor's research is synopsized in reports which are filed with the Defense Technical Information Center (DTIC). A list of contracts in place during FY 82 is included as Appendix C. Readers desiring specific contract report should make request to DTIC.

VII. QUESTIONS:

Questions or comments about this report are welcomed and may be addressed to:

Commander
USAMRIID
Fort Detrick
Frederick, Maryland 21701

AUTHOR INDEX

ANNUAL PROGRESS REPORT - FY 82

TABLE OF CONTENTS

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION: DAOG 1519	2. DATE OF SUMMARY: 82 10 01	REPORT CONTROL SYMBOL: DD-DR&E(AR) 636
3. DATE PREV SUMRY: 81 10 01	4. KIND OF SUMMARY: D. CHANGE	5. SUMMARY SCTY: U	6. WORK SECURITY: U	7. REGRADING	8. DISB'N INSTR'N: NL	9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AN	200
b. CONTRIBUTING				
c. CONTRIBUTING	STOG-80-7.2:2			

11. TITLE *(Precede with Security Classification Code)*
(U) Characterization of Microbial Toxins of Potential BW Importance

12. SUBJECT AREAS
003500 Clinical Medicine; 004900 Defense; 012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	7.9	361
c. TYPE	d. AMOUNT	83	8.0	491
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME: USA Medical Research Institute of Infectious Diseases	a. NAME: Pathology Division, USAMRIID
b. ADDRESS *(Include zip code)*: Fort Detrick, MD 21701-5011	b. ADDRESS: Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL: Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR: Middlebrook, J
d. TELEPHONE NUMBER *(Include area code)*: 301-663-2833	d. TELEPHONE NUMBER *(Include area code)*: 301-663-7211
21. GENERAL USE: FIC MILITARY/CIVILIAN APPLICATION:	f. NAME OF ASSOCIATE INVESTIGATOR *(If available)* g. NAME OF ASSOCIATE INVESTIGATOR *(If available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Microbial Toxins; (U) Biochemistry; (U) Therapy; (U) Toxoids; (U) Laboratory Animals

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Study staphylococcal enterotoxin C (SEC), botulinum toxin, Pseudomonas aeruginosa exotoxin A, diphtheria toxin, anthrax toxin and T-2, to develop prophylactic or therapeutic measures, for the protection of U. S. military personnel.
24. (U) Toxin structure will be studied (possible synthetic vaccine) and their synthesis [possible nontoxic mutant toxins (CRM's) for vaccines]. Then, target cell work will be done. The toxin-induced biophysical and biochemical events which lead to the cell/tissue response will be studied along with test drugs to find those which may protect the cell/tissue from the toxin; and test positive findings in laboratory animals to determine human therapeutic potential.
25. (U) 8110-8209 - The area within the disulfide bond of SEC was obtained by reaction of the protein with nitrothiocyanobenzoic acid and sequenced. Acetylcholine release and calcium uptake and release assays are operational for PC12 and NG108-15 cell lines and for synaptosomes isolated from guinea pig cortex. Sodium-calcium exchange, ATP-stimulated calcium uptake and Ca2+ efflux assays are operational for inverted synaptic plasma membrane vesicles. Dose-Response relationships have been established for the effects of botulinum toxin type A on acetylcholine release from synaptosomes. Preliminary results suggest botulinum type A inhibits acetylcholine release from the PC12 nerve cell line. Other results suggest botulinum type A affectscalcium metabolism in both the PC12 cell line and in inverted synaptic plasma membrane vesicles. Fourteen T-2 resistant cloned CHO cell sublines were obtained and it was shown that at least 4 of the T-2 resistant sublines were toxin binding and/or uptake mutants. The T-2 resistant cells were demonstrated to be cross-resistant to several other trichothecene toxins. Several kinetic and equilibrium parameters of T-2 cell association were determined or transport sytstem for T-2. This suggested the existence of a plasma membrane receptor or transport system for T-2.

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)

Work Unit No. S10 AN 200: Characterization of Microbial Toxins of Potential BW Importance (U)

Background:

Toxic proteins play an important role in the pathogenesis of many bacterial infections. Cholera, diphtheria and botulism are examples of bacterial disease in which the bacterial toxin is solely responsible for the clinical symptoms. There are other examples such as Pseudomonas, E. coli and anthrax where protein toxins are apparently involved but their degree of importance is not firmly established. Clearly, in all such diseases, successful treatment and/or prevention of the disease must deal somehow with the toxin.

With certain microbial toxins, there is also a concern with their use as BW agents. Botulinum toxin for example, is one of the most poisonous substances known to man. Moreover, it is a relatively easy task to prepare large quantities of the toxin making its potential use a real threat. Likewise, the staphyloccocal enterotoxins are potential BW agents and could be obtained without too much effort or technology. Recent events in Southeast Asia have led to a growing concern that certain small molecular weight toxins have or may be employed as BW agents. These revelations have resulted in a considerable scientific effort by USAMRIID in which we have played a small role.

The principal function of this work unit is to study and characterize microbial toxins with an ultimate aim towards the development of protective measures. To meet this goal we are employing a two-pronged approach. First, we are studying several of those toxins proven to be potential BW agents as regards structure, mechanisms of action, etc. Secondly, based on a large amount of evidence that most protein toxins have several common features to their mechanisms of action, we have employed a well characterized model (diphtheria toxin) to study certain basic questions, the answers to which we believe would have major impacts on our efforts with the accepted BW agents.

Progress:

1. Botulinum toxin. Two nerve cell lines, PC12 (rat pheochromocytoma) and NG108-15 (mouse neuroblastoma X rat glioma hybrid) were chosen for the botulinum toxin (Botx) project based upon their ability to release acetycholine (ACh) and form synapses analagous to those at neuromuscular junctions. Cultured cell lines are the only system currently available that provide sufficient homogeneous cholinergic material for biochemical studies of toxin mode of action. Earlier studies of ACh release in response to stimuli employed U-shaped tubes which are unsuitable for our purposes; it was necessary to adapt the ACh release assay to 24-well culture plates. Poly-l-lysine enhanced adhesion of the cells to the plates and allowed vigorous washing of the wells without significant cell loss. Acetylcholine release was determined by selective extraction of tritiated ACh from the cell supernatant and subsequent liquid scintillation techniques. Serum "factors" substantially influence ACh release, complicating this portion of the study. We now screen sera prior to ordering and supplement sera with controlled concentrations (50 ng/ml) of

nerve growth factor (NGF) for one to two weeks before plating the cells. In the near future, serum-free media will be tested and, if found suitable for our studies, employed routinely. We have found NGF to be essential to the ACh release assay. The result of exposure of the cells to NGF in the growth medium is a reduced spontaneous release of ACh, thereby providing a greater ratio of stimulated to non-stimulated release. This allows us to determine more precisely the effects of toxic agents on the system. Typical ratios of stimulated to nonstimulated release are 2:1 (stimulation with veratridine) and 5:1 (stimulation with either 80 mM KCl or 1 mM carbamylcholine). Studies paralleling the cell culture work were conducted using synaptosomes isolated from guinea pig cortex. Although this system is a heterogeneous population of cell types, it is suitable for limited applications in the studies of Botx. We have adapted our method of ACh detection to this system and have obtained results of acetylcholine release (in response to elevated KCl concentrations) in excellent agreement with the literature. Table I shows data derived from a single experiment in which PC12 cells were incubated in the presence or absence of Botx (1000 mouse LD_{50}/ml) in the bathing medium for 90 min. Although these results verify earlier studies, problems with the cell culture (contamination), incubators (CO_2 and temperature control failure) and sera have prevented us from defining exactly the conditions for these effects. The results below demonstrate a block of stimulated acetycholine release by Botx.

TABLE I. ACh RELEASE

	fmol ACh Released / g protein x 10 min X ± S.E. (4)	
Condition	Control	Botx-treated
Nonstimulation (5.4 mM K+)	15 ± 1.6	18 ± 2.2
Stimulation (80 mM K+)	34 ± 2.2	21 ± 2.3

Similarly, we have demonstrated (Table II) a 70% block of stimulated ACh release from synaptosomes incubated with 107LD_{50}/ml Botx type A (90 min) in close agreement with the literature.

TABLE II. ACh RELEASE

	CPM: X ± S.E (3)	
Condition	Control	Botx-treated
Nonstimulation (2.7 mM K+)	2520 ± 28	2230 ± 29
Stimulation (20 mM K+)	3990 ± 240	2960 ± 61

Calcium uptake studies have been undertaken with the cell lines and synaptosomes. In control studies, calcium uptake increases with time of depolarization, paralleling ACh release as expected. Using the same experimental protocol as in Table I, but assaying for Ca^{++} uptake by PC12 cells, we obtained the data shown in Table III.

TABLE III. CALCIUM UPTAKE

	CPM: X ± X.E. (4)	
Condition	Control	Botx-treated
Nonstimulation (5.4 mM K+)	480 ± 67	540 ± 38
Stimulation (80 mM K+)	1130 ± 92	850 ± 43

The above results suggest calcium metabolism may be affected by Botx. Studies employing the nerve cell lines and synaptosomes expose the toxin to the outer (extracellular) leaflet of the plasma membrane bilayer. In contrast, inverted synaptic plasma membrane vesicles expose the toxin to the inner (cytoplasmic) leaflet of the bilayer. In addition, this system allows the effects of the toxin on membrane-mediated calcium metabolism to be determined. The processes of Na-Ca exchange, ATP-stimulated calcium uptake (interpreted as in vitro calcium extrusion from cells) and calcium release (uptake into cells in vivo) can be observed. Therefore, we have isolated inverted membrane vesicles using established procedures and obtained calcium uptake curves in good agreement with published results. Preliminary results with this preparation suggest Botx affects membrane-mediated calcium metabolism.

We have employed beta-bungarotoxin (B-Butx), a presynaptically-acting toxin from the venom of the snake Bungarus multicinctus, as a positive control in our studies. This toxin, although much less toxic to animals than Botx, inhibits ACh release in a biphasic manner. Beta-bungarotoxin causes an initial increase in neurotransmitter release followed by a decrease and, finally, a total block of transmitter release. Several reports of interactions between Botx and B-Butx have appeared in the literature. It has been proposed that B-Butx increases intracellular calcium concentrations although no direct evidence for this effect has appeared in the literature. We have shown that B-Butx causes an initial (within 15 min) stimulation of spontaneous ACh release from synaptosomes, in agreement with the literature. We have also shown that B-Butx increases calcium uptake into synaptosomes (a new finding). In addition, we found that B-Butx inhibited Na-Ca exchange in inverted synaptic plasma membranes and interpret these results as suggesting B-Butx blocks calcium extrusion from the nerve terminal, thereby greatly increasing calcium concentrations in the cytoplasm.

Work was also begun on the purification of type A botulinum neurotoxin in order to evaluate possible methods to obtain material of sufficient homogeneity for structural studies. The starting materials for this study were several batches of "crystalline" toxin prepared three years ago by Dr. L. Siegel of our Division. The "crystalline" toxin is actually a mixture of hemagglutinin, neurotoxin, and other proteins, and may be as much as 90% hemagglutinin. In contrast to previously published methods, we found that the neurotoxin did not bind to diethylaminoethyl anion exchange resins (DEAE-cellulose and DEAE-sephacel). Nonetheless, a certain

degree of purification was effected, although the product remained contaminated with small amounts of two other proteins. Consequently, other chromatographic methods are being explored to remove the last contaminants. These include carboxy-methyl sepharose, hydroxyl apatite, and three types of NAD-agaroses. Of these, the first two appear to be the most promising as judged by biological assays. Electrophoretic studies are currently in progress.

Since Dr. B. R. DasGupta of the University of Wisconsin has a contract from the U.S. Army for research on the protein chemistry of botulinum neurotoxins, we felt that a collaborative effort would be in the best interest of both laboratories with respect to structural studies of types A and E. We therefore arranged that certain polypeptides from the neurotoxins would be prepared by Dr. DasGupta and his associates, and sent to this laboratory for partial amino acid sequencing; we recently received samples of the heavy and light chains of type A. After chemical modification and toxicity testing, now underway, these peptides will be placed in the automatic sequencer. The amino terminal region of one of these chains and the carboxy-terminal region of the other encompass the site of "nicking" by proteases of type A in culture. It is important to obtain the structure of this region since it represents an external protein-protein interaction site and, as such, it is a good candidate for synthetic antigen production.

2. T-2. Efforts with T-2 followed two main themes: (a) studies with wild-type cells and (b) the development and characterization of T-2 resistant cells. It was hoped that by studying the molecular mechanism of action of T-2 with wild-type cells and comparing the results with similar experiments using resistant cells, that we might uncover some stage or step that could be exploited in a protective sense. It is entirely possible that we have already achieved our goal as will be discussed below.

To develop T-2 resistant cells, we employed the Chinese Hamster Ovary (CHO) cell line. These cells were treated with 250 mg/ml of the mutagen ethyl methane sulfonate (EMS) for 16 h, followed by a wash to remove dead or dying cells and the drug. After a 20 h further incubation to allow phenotypic expression of the (presumed) mutations, cells were challenged with concentrations of T-2 ranging from 0.1-10 μg/ml. After 24 h, the toxin was washed out and the cells incubated for the development of resistant colonies. Although several colonies did develop when tested, none proved to be resistant to T-2.

We repeated the entire process altering our procedure to leave the T-2 in with the cells for a period of several weeks. From this experiment there developed one T-2 resistant isolate which was cloned by limiting dilution. Fourteen T-2 resistant sublines designated CHOT2R1, CHOT2R2, etc., were obtained and frozen to ampules to assure seed stocks. One of these lines has been in continuous culture for over 5 months with no detectable loss of resistance. This time frame, with the split ratio we employ, means that the original cloned cell has undergone somewhere between 500-800 divisions suggesting a stable mutation. Before describing our studies into the nature of the mutation(s) responsible for T-2 resistance, it is appropriate to outline the results from our concurrent studies with wild-type CHO cells.

At the outset, we were aware that the literature claimed T-2 inhibits protein synthesis during polypeptide chain initiation. Therefore, we examined the effect of T-2 on protein synthesis in CHO cells. After an incubation of only 1 h, we detected a slight (10-20%) inhibition of protein synthesis of 0.0008-0.1 μg/ml T-2; further incubation did not appreciably alter this dose-response curve. Using other

trichothecene toxins supplied by Physical Sciences Division, we determined their protein synthesis inhibitory potencies (CHO cells) to be in the order roridin A verru-carin A >T-2 ≃HT-2 > T-2 triol ≃ T-2 tetraol ≃ deoxynivalanol.

When radiolabeled T-2 became available (courtesy Dr. Pace), we initiated a series of experiments to measure various biophysical parameters of T-2 cell association. Our assay was simply to add labeled toxin to cells in 24 well plates and, after the times desired, rinse the monolayer well, add 0.1 M NaOH to dissolve the cells and count an aliquot of the base in aquasol. The kinetics of T-2 cell association at both 4 and 37° exhibited typical bimolecular reaction patterns, increasing to and maintaining a steady-state plateau. At 4°, equilibrium was reached in approximately 6-8 h (0.01 μg/ml T-2) whereas, at 37° equilibrium was attained by 1-1 1/2 h. Not only were the kinetics of T-2 cell association more rapid at higher temperatures, the equilibrium state (and therefore, the total number of toxin molecules bound and/or transported) was 4-5 fold higher at 37° than 4°.

When we included unlabeled T-2 at a 100-fold molar excess in our kinetic experiments, toxin-cell association at both 4 and 37° was blocked by 95-98%. This observation is especially significant because it implies that the plasma membrane acts as a barrier to intracellular transport of T-2. If T-2 entered target cells by simple diffusion, there should be no competition because diffusion is not a saturable process. What is more, when we compared the uptake of α T-2 (the naturally occurring and biologically active form) with β T-2 (the enantiomer and biologically inactive form), we found that the α isomer was taken up by CHO cells at a rate 50 times that of the β. This result suggests a steriospecificity of the uptake process, a feature certainly not expressed by diffusion.

By varying the amount of radiolabeled T-2 added, and keeping the binding time constant, we obtained binding isotherms. This data could be processed by the method of Scatchard to yield the so-called "Scatchard plot" from which we estimated the disassociation constant for T-2 cell binding to be 2.6×10^{-8} M and the number of binding sites per cell at $3\text{-}6 \times 10^{6}$ (both at 4°). In a slightly different approach we added a constant amount of radiolabeled T-2 and varying levels of unlabeled T-2. The concentration where radiolabel binding is blocked 50% corresponds to the disassociation constant which, in our case, was 3×10^{-8} M, a value in good agreement with the Scatchard derived estimate.

Returning now to studies with resistant cells, we used the protein synthesis assay to test our mutagenized and cloned cells for resistance. A 2.5-3 fold resistance was observed with all the lines cloned and frozen as seed stock. While not a large factor of resistance, the effect was very easy to document and was more than adequate to allow several interesting lines of study.

For one thing, we noted that the dose-response curve for T-2 inhibition of DNA synthesis was shifted by exactly the same factor as that for protein synthesis. This observation struck us as odd because the literature indicated T-2 directly inhibited DNA synthesis. Obviously, if this were true, we had obtained resistant cells with 2 mutations, one in the DNA and one in the protein biosynthetic machinery. Since our approach should have yielded a productive mutation at a rate not much greater than 1 in 10^{6} and since the change of a double mutation is the product of the singles (1 in 10^{12}), we were dealing with a very rare cell indeed. An alternative explanation is that direct inhibition of protein synthesis led indirectly to inhibition of DNA synthesis. We tested cycloheximide and puromycin, well established to act on protein synthesis, for their effects on RNA and DNA

synthesis. Qualitatively and quantitatively, we obtained the same effects with these drugs as with T-2. Therefore, we conclude that contrary to literature claims, T-2 does not directly inhibit DNA synthesis. It follows that if we can protect from T-2 inhibition of protein synthesis, effects on DNA synthesis will be ameliorated.

A second and extremely important point brought out by the T-2 resistant CHO cells is their pattern of cross-resistance to other trichothecene toxins. When tested, the T-2 resistant mutants proved resistant to roridin A, verrucarin A, H-T2, T-2 triol and deoxynivalanol; cross-resistance to T-2 tetraol was not observed. Interestingly, if we used these trichothecene toxins to block radiolabeled T-2 cell association we found roridin A, verrucarin A and T-2 were very effective competitors, T-2 triol and deoxynivalanol were much less potent and T-2 tetraol had little or no effect. This pattern, in conjunction with the cross-resistance cited above, provides strong evidence for a specific but common bind site for all the above but T-2 tetraol. We would propose that development of agents which interfere with the binding step discussed above would be particularly attractive since protection from several of the trichothecenes should result.

3. Anthrax. Amino acid analyses were done on the "PA" component of anthrax toxin, produced under contract by the Michigan Department of Public Health. Samples were hydrolyzed with and without prior oxidation with performic acid. Results showed extensive differences when compared to earlier analyses of PA prepared here by Dr. S. Leppla, particularly with respect to the content of alanine, leucine, lysine, glutamic acid, and methionine. Cysteic acid was recovered from performic acid-oxidized samples, but the amounts were too low to quantitate accurately. However, the half cystine content can be estimated at about two to three residues per mole. When the hydrolysate was rerun on a second amino acid analyzer under different chromatographic conditions, an unknown peak emerged just before valine with an elution volume that is identical to that for glucosamine. These observations suggest that the PA preparation may not be completely pure.

Finally, two preparations of PA were placed in the automatic sequencer. The first, from the Michigan Department of Health, did not yield interpretable data, but supported the possibility that this material is not completely pure. The second preparation, from Dr. Leppla, was either non-uniform in the amino terminal region or was contaminated with small amounts of other proteins. Multiple phenyl-thiohydantoin derivatives were obtained at each step, but a very short tentative sequence could be deduced. Further structural characterizations of PA await and depend on the future availability of a sufficient quantity of homogeneous material.

4. Staphylococcal Enterotoxins. It was found that limited proteolysis of staphylococcal enterotoxin C (SEC) by trypsin produced five peptides, not three as previously reported, i.e., two small peptides were overlooked in the original isolation procedure.

The purification and sequencing of the two extra peptides was described in the preceding annual report. The first, a dipeptide, was placed in the overall structure of SEC by virture of homology with the corresponding area of staphylococcal enterotoxin B (SEB). The second, a pentapeptide, showed little homology with any area of SEB, but evidence suggested that it should be placed within the disulfide loop of SEC.

Isolation of an overlap peptide was necessary, and this was accomplished by cleavage of intact SEC at the half cystine residues. The reagent 2-nitro-5-

thiocyano benzoid acid (NTCB) was synthesized and reacted with SEC according to published procedures. The resulting peptide mixture was purified by chromatography on Sephadex G-50. While the desired peptide could be obtained in this way, it was found that the lysine residues had been carbamylated by the reagent. This observation has not been previously reported in the literature.

To produce the peptide in an unmodified form, an alternative procedure was devised using sodium cyanide and 5-5'-dithio-bis-2-nitro benzoic acid (DTNB). The products were purified on Sephadex G-50 and by high pressure liquid chromatography. Amino acid analysis and sequencing showed that the tentative location of the penta-peptide was correct.

The last area of SEC investigated was the cyanogen bromide cleavage site near the carboxy-terminal end of the molecule. It was recognized that production of an overlap peptide was necessary to check for the presence of a double methionine sequence at this site. This was accomplished by reacting intact SEC with hydroxylamine, and the products were purified on Sephadex G-50. The large peptide containing the area in question was then sub-fragmented with chymotrypsin, and the mixture was purified by high pressure liquid chromatography. The digest was more complex than expected, but the following peptides were found: (a) met tyr, (b) met met tyr, and (c) leu met met tyr. These data confirmed the double methionine sequence in this area of SEC.

Presentations:

Middlebrook, J. L. Isolation and preliminary characterization of T2-resistant mammalian cells. Presented International Congress of Biochemistry, Perth, Australia, 15-22 August 1982.

Publications:

1. Middlebrook, J. L. and D. L. Leatherman. 1982. Differential sensitivity of reticulocytes to nicked and unnicked diphtheria toxin. Experimental Cell Res. 138:175-182.

2. Dorland, R. B. 1982. The protective mechanism of action of amines in diphtheria toxin treated Vero cells. Can. J. Microbiol. 28:611-617.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DAOG 1522	82 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
81 10 01	D. CHANGE	U	U		NL	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AO	199
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80:7.2:2			

11. TITLE *(Precede with Security Classification Code)*
(U) Bacterial and Rickettsial Diseases of Potential BW Importance

12. SUBJECT AREAS
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	13.8	706
c. TYPE	d. AMOUNT	83	15.0	921
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Bacteriology Division, USAMRIID
b. ADDRESS *(include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR Hedlund, K W
d. TELEPHONE NUMBER *(include area code)* 301-663-2833	d. TELEPHONE NUMBER *(include area code)* 301-663-7341
21. GENERAL USE FIC MILITARY/CIVILIAN APPLICATION:	f. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Scott, G g. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Lowry, B S

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Bacterial Diseases; (U) Q Fever; (U) Laboratory Animals

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Study well-recognized and newly discovered pathogens wih acknowledged BW potential, requiring special containment. Assess and characterize virulence factors, growth requirements and pathogenesis in animal models and resultant therapeutic implications, to provide a base for developing vaccines to protect U.S. military personnel.

24. (U) Current approaches are sophisticated and broad in scope and are not limited to these examples: isolation and characterization of bacterial plasmids that regulate toxin production; purification and characterization of antigenic components of Coxiella Burnetii and characterization of sequential host response to aerosolized microbial pathogens.

25. (U) 8110 - 8203 - Chemical characterization of purified soluble phase I antigen of Coxiella burnetii was obtained. An anti serum agar method for simultaneous detection and isolation of Legionella species was published. Plasmids have been discovered and partially characterized in B. anthracis which regulate the production of all the anthrax toxin components (PA, LF, EF). It has been discovered that when the plasmids of B. anthracis are cured the production of all 3 toxin components cease. An ELISA assay for titration of antibodies to C. burnetii and for the detection of C. burnetii antigens in infected mouse spleen tissue has been developed. Studies have begun to determine suitability of inbred A/J mice as the animal model for evaluating inactivated Q fever vaccine. A new fluorescent antibody technique was used to follow the progression of aerosolized sublethal doses of Legionella pneumophila in guinea pigs. It was less than successful because there was not sharp histologic step wise progression of pneumonic legionellosis but a blurred overlapping picture in which the exact time sequence is lost. Publications: Acta Virol 25:241, 1981; J. Clin. Microbiol. 13:115, 843, 1981; Ann. Int. Med. 94:413, 1981; Pharmacol. Exp. Ther. 15:123, 1981; Infect Immun. 31:1270, 1981; J. Infect Dis. 143:562, 1981.

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)

Work Unit No. S10 A0 199: Bacterial and Rickettsial Diseases of Potential BW Importance

Background:

This work unit consists of five research units which will be cited individually. They will be grouped as to areas of interest rather than numerically.

Dr. Wachter is terminating his research work unit. The objectives of this research unit were (a) to determine the potential of the soluble phase I antigen of Coxiella burnetii for use as a vaccine, and (b) to obtain characterization data on the extracted antigen to determine its possible use as a "subunit" vaccine.

The antigen, which had been tested as a vaccine in human volunteers in Czechoslovakia and Romania (1, 2), had been shown to be much less reactogenic than either cell-wall or whole organism preparations of C. burnetii (3, 4). Anacker et al. (3) reported that an average dose of 5 micrograms of the antigen as the trichoroacetic acid (TCA) extract protected 50% of guinea pigs against a challenge of 1×10^4 ID_{50} of the California strain. In earlier studies, TCA extract preparations of the antigen contained polysaccharide, protein, and lipid (4, 5, 6), i.e. an uncharacterized mixture rather than a purified single antigen.

Dr. George H. Scott is evaluating animal models for Coxiella burnetti.

Coxiella burnetti is among the most infective of the pathogenic rickettsiae. Its host range encompasses many wild and domestic mammals and birds as well as some arthropods. However, overt disease and death rarely occur in experimentally infected laboratory animals unless massive doses of the rickettsia are injected. Diagnosis and monitoring of the infection in guinea pigs must rely on serologic conversion, histologic alterations, biochemical changes (7, 8), and isolation of the rickettsiae. Unfortunately variations associated with these observations limit their value for precise evaluations of prophylactic and therapeutic measures.

Several investigators have demonstrated the influence of genetic background on the experimental host's resistance to infectious disease (9, 10). Under this work unit, an array of genetically defined inbred mice, hybrid mice, and other selected species are being examined in search of an immunocompetent animal in which Q fever is manifested through quantifiable overt illness or death. This work was motivated primarily by the need for an animal model against which to gauge the protective efficacy of Q fever vaccines. Therefore, animals with known immunologic deficiencies have not been included.

Mr. Janssen and LTC Lowry have terminated the research work unit which dealt with the diagnosis and pathogenesis of Legionnaires' Disease. Research was intended to improve methods for the detection and isolation of Legionella species in clinical and environmental specimens; to identify and control potential sources of Legionnaires' Disease and Pontiac Fever; to define the pathogenesis of legionellosis; and to develop and improve study techniques.

A second research work unit under LTC Lowry's direction deals with infectivity of aerosolized Legionella organisms. The thrust of this research unit is to develop a surgically altered guinea pig model and compare variations in respiratory tract-vascular system deliverance of infectious organisms and explore possible modes of intervention in the disease progression; both for strains yielding pulmonic legionellosis and those considered to cause the non-pulmonic (Pontiac Fever) disease.

Finally, Dr. Knudson, Captains Mikesell and Dreier directed the detection and characterization of plasmids and pathogens of military importance. Multiple drug resistant plasmids complicate the treatment of infectious diseases by rendering their bacterial hosts resistant to specific antimicrobial agents. This drug resistance can be conjugally transferred in vivo to other bacteria even across species lines. Virulence plasmids specify properties that directly contribute to the pathogenicity of the bacteria, such as plasmid-encoded toxins and adherence proteins. Extrachromosomal genetic determinants code for some botulinus toxins, diphtheria toxin, streptococcus erythrogenic toxin, and the enteropathogenic Escherichia coli enterotoxins and pilus-like colonization antigens. It is feared that certain molecular genetic manipulations using plasmids as vectors could allow the design and construction of new species of pathogens against which our present methods of identification and treatment will be ineffective.

This research work unit is designed to establish a technological base for the genetic analysis of pathogens and to help evaluate the BW threat from pathogens which contain either naturally occurring plasmids or recombinant DNA molecules. Methods have been developed for the rapid detection, isolation and characterization of extrachromosomal DNA. These methods were successfully applied to the genetic analysis of several pathogens of military importance. This research led to the discovery of plasmids in Legionella pneumophila (11) and related species of Legionella (12), and in Bacillus anthracis where the plasmid was shown to be associated with toxin production (13).

Progress:

By comparison with dextrans of known molecular weights in gel filtration on Sepharose-2B the molecular weight of the soluble phase I Coxiella burnetti antigen was estimated to be 600,000 daltons.

Gravimetrically it was determined that 1 ml of TCA extract (195 g dry weight) would yield 8 g of column-purified phase I antigen, i.e., CF^+ component. Based on the expected yield of purified phase I C. burnetti per yolk sac (14) the yield of purified phase I antigen would be 1.4 µg per yolk sac.

In skin tests using sensitized guinea pigs, the purified antigen induced mild induration and erythema of short duration in some animals. These effects were 5 to 10 times less severe than those produced by TCA extract preparations in the same guinea pigs. Therefore, the purified antigen is 500 to 1,000 times less reactogenic than the intact organism.

In current tests using a micro-slide double diffusion technique a single precipitin line has been observed between the purified antigen and guinea pig hyperimmune serum. Also, during this report period a collaborative program was initiated with Dr. Robert Seid, WRAIR, to determine the chemical composition of the column-purified phase I antigen of C. burnetii. The CF^+ fractions from a number of fractionations using Sepharose-2B were pooled, dialyzed extensively against distilled water, concentrated by pervaporation and lyophilized.

Samples for amino acid and amino sugar analysis were suspended in concentrated hydrochloric acid, hydrolyzed in vacuo for 24 h at 105°C, evaporated to dryness, then reconstituted in pH 2.2. Identification and quantitation by comparison with known standards was accomplished with a single microbore amino acid analyzer (15, 16). The amino acid composition of the antigen (Table I), was characterized by a high content of lysine (21.7%), serine (19.9%) and glutamic acid (17.7%), and an absence of proline and relatively low level. The low concentration of the aromatic amino acids, tyrosine and phenylalanine, might account for the low UV-absorbance of the purified antigen and its lack of response in the Lowry protein determination. By contrast, when the cell envelope of phase I C. burnetii were examined by Jerrells et al. (17), relatively high concentrations of tyrosine and phenylalanine, proline, and arginine were detected and the most abundant amino acids are alanine, glutamic acid, aspartic acid, and glycine.

For the determination of neutral monosaccharides, antigen samples were suspended in a mixture of methanesulfonic acid and Dowex 50-X8 resin for identification and quantitation by liquid chromatography (18, 19). The neutral monosaccharides of the antigen were comprised of approximately 80% glucose and heptose (Table II). The value for glucose very probably includes a contribution by mannose. Mannose has been identified as a major constituent of purified LPS from C. burnetii, phase I which was obtained by phenol extraction of rickettsia (20). Previous studies by using paper chromatographic procedures, Jerrells et al (17), identified glucose, galactose and glucuronic acid in the cell envelope of phase I C. burnetii.

TABLE I. AMINO ACID AND AMINO SUGAR COMPOSITION OF PHASE I ANTIGEN OF C. BURNETII

AMINO ACID	MOL/MG	PERCENT
Aspartic acid	0.117	3.98
Threonine	0.178	5.44
Serine	0.737	19.86
Glutamic acid	0.469	17.70
Proline	0	0
Glycine	0.452	8.70
Alanine	0.233	5.31
Valine	0.103	3.09
Methionine	0.007	0.28
Isoleucine	0.048	1.63
Leucine	0.072	2.42
Tyrosine	0.030	1.39
Phenylalanine	0.034	1.44
Glucosamine	0.065	2.97
Lysine	0.579	21.70
Histidine	0.103	4.11
Arginine	0	0
Unknown[a]		

[a]Unknown compound noted but not quantified.

TABLE II. NEUTRAL MONOSACCHARIDE COMPOSITION OF THE PHASE I ANTIGEN OF C. BURNETII

MONOSACCHARIDE	MOL/MG	PERCENT
Rhamnose	0.055	3.61
Fucose	0.109	7.13
Ribose	0.152	9.07
Glucose[a]	0.789	56.60
Heptose	0.306	23.60
Dideoxyhexose[b]		

[a]Mannose also elutes in this position.
[b]Peaks eluting in dideoxyhexose position noted but not quantified.

Samples for fatty acid analysis were hydrolyzed with 4 N hydrochloric acid at 100°C for 4 h under nitrogen, diluted with water, and extracted three times with benzene/diethyl ether (1:3). The organic layer was washed twice with water before drying over sodium sulfate. The fatty acids were converted to methyl esters with diazomethane (21) before injection into a Hewlett-Packard gas chromatograph. Separation was effected on a glass column containing 3% SP-2100 DoH on 100/200 Supelcoport (Supelco, Inc., Bellefonte, PA), with a temperature program of 140° to 225° at a rate of 4° per min. Resultant peaks were identified with known standards.

The fatty acid composition of the antigen is shown in Table III. Two straight-chain fatty acids were the most abundant: palmitic (32.1%) and stearic (22.3%). In contrast, Chan et al. (20) reported that the major fatty acids of the total extractable lipids of whole organism phase I C. burnetii were the branch-chain sarcinic (33.1%) and 14-methylhexadecanoic (34.1%). In the phase I antigen these represented only 5.8% and 11.4%, respectively. Composition of the antigen consisted of 25.1% carbohydrate, 39.0% protein and 8.6% fatty acids. Contributions by dideoxyhexose (Table II) and an unknown amino acid (Table I) would raise carbohydrate and protein values slightly; however, totality of the components in Table IV failed to account for 100% of the antigen. This finding was analogous to previous observations of lipopolysaccharide content reported by Chan et al. (20) of 50-80% and Schramek and Brezina (22) of 60.2%.

Dr. G. H. Scott has screened 46 strains of inbred mice in search of immunocompetent animals in which C. burnetii infection is manifested through quantifiable overt illness or death. Ten 7-week old female mice from each strain were inoculated by the intraperitoneal (i.p.) route with $10^{6.5}$ $GPIPID_{50}$ of the Nine-Mile strain of C. burnetii, phase I. The mice were observed for overt signs of illness and death for a period of 28 days. Surviving mice were bled, their sera pooled and assayed for anti-C. burnetii antibodies using IFA procedures.

Only 14 of the 46 strains that were tested exhibited overt signs of illness evidenced by rough hair coats and lethargy. These are listed in Table V together with data on the duration of illness, the mortality rate by strains, and the antibody titers in surviving mice.

TABLE III. FATTY ACID COMPOSITION OF PHASE I ANTIGEN OF C. BURNETTI

FATTY ACID	PERCENT
C_{14}(myristic)	0.87
iso-C_{15} (sarcinic)	5.80
C_{16} (palmitic)	32.10
iso-C_{17} (methyl hexadecnoic)	11.40
$C_{18:1}$ (oleic)	12.50
C_{18} (stearic)	22.30
C_{20} (arachidonic)	6.90
C_{22} (behenic)	4.90
-OH-C_{14} (-hydroxymyristic)	3.20

TABLE IV. COMPOSITION OF PHASE I ANTIGEN OF C. BURNETII

CONSTITUENT	PERCENT
Carbohydrate	25.1
Protein	39.0
Fatty acids	8.6
KDO[a,b]	
Phosphorus[b]	

[a]2-keto-3-deoxyoctonate.
[b]To be determined.

TABLE V. RESPONSES OF SELECTED INBRED MICE TO I.P. INOCULATION WITH $10^{6.5}$ $GPIPID_{50}$ OF THE 9-MILE STRAIN OF C. BURNETII, PHASE I

	OVERT ILLNESS				
STRAIN	PERCENT	AVERAGE DURATION, DAYS	RANGE, DAYS	DEAD/ TOTAL	IFA[a] TITER
A/J	95	4.0	4-16	9.20	1280
A/HeJ	100	3.5	6-12	0/10	640
A/WySnJ	90	6.5	4-12	3/10	1280
Au/SsJ	40	7.8	5-15	3/10	640
BAlb/CDub	100	4.2	4-12	0/10	1280
BAlb/CJ	100	7.0	3-12	0/10	2580
BAlb/cNcrdBr	100	-	6-28	0/10	2580
BAlb/cByJ	90	10.4	4-18	0/10	1280
C3H/HeJ	50	3.1	6-9	0/10	640
NzB/BinJ	40	2.6	7-9	2/10	640
LG/J	20	3.0	6-8	0/10	2560
129/SvJ	90	5.9	4-15	0/10	2560
129/J	90	8.3	4-22	0/10	1280
BDP/J	70	3.6	7-9	0/10	ND

[a]Pool of sera from survivors.

Essentially all mice in substrains of the A, Balb/c, and 129 lines became overtly ill within 3 to 6 days after inoculation, and the illness persisted from 6 to 22 days. However, in spite of the high dose of rickettsia administered, mortalities occurred in only 4 strains; the greatest number of deaths (45%) occurred in strain A/J.

Listed in Table VI are 32 strains that we have designated as non-responders. Although the rickettsia apparently replicated in these strains, as evidenced by elevated antibody titers, the mice remained healthy and vigorous throughout the 4-week post inoculation period.

TA3LE VI. NON-RESPONDERS; INBRED STRAINS OF MICE THAT EXHIBITED NO OVERT SIGNS OF ILLNESS FOLLOWING I.P. INJECTIONS OF C. BURNETII

STRAIN	IFA[a] TITER	STRAIN	IFA[a] TITER	STRAIN	IFA[a] TITER
AKR/J	2560	CBA/CaJ	1280	CBA/HT6J	2560
C57BL/6J	2560	C57BL/10J	2560	C7BL/SnJ	1280
C57BL/KsJ	1280	C57BR/CdJ	320	C58/J	1280
C3HeB/FeJ	2560	C3H/HeDub	640	C3H/RV	1280
DBA/1J	1280	DBA/2J	2560	C3H/HeSnJ	1280
C57L/J	640	CBA/J	2560	C57BL/6BYJ	2560
ILn/J	1280	DBA/2fMaiBR	1280	C3H/B1MaiBR	640
C57/6MaiBR	2560	St/bj	2560	RF/J	2560
R111S/J	1280	SEC/RcJ	1280	SJL/J	2560
MA/MyJ	1280	SWR/J	1280	PL/J	ND
CE/J	ND	SEA/CNJ	ND 1		

[a]Pool of sera from survivors.

No consistent differences between antibody titers in the non-responders and in strains that became overtly ill and/or died were observed.

Studies were initiated to further characterize the susceptibility of several of the more sensitive strains that were identified by these screening tests, and to examine the influence of mouse age and sex on their susceptibility. Estimates of the median infective doses (ID_{50}) estimated serologically, the median effective dose (ED_{50}) based on overt illness, and the median lethal dose (LD_{50}) for three groups of A/J mice, and strains A/WySnJ, Balb/cJ, and 129/J are presented in Table VII.

TABLE VII. MEDIAN I.P. DOSE OF C. BURNETII FOR SELECTED STRAINS OF INBRED MICE

STRAIN	MOUSE AGE AND SEX	LOG_{10} GPIPID$_{50}$ ID_{50}	ED_{50}	LD_{50}
A/J	7 wk, F	1.4	5.2	6.2
A/J	7 wk, M	1.7	5.8	7.6
A/J	4 wk, M	1.7	6.2	7.6
A/WySnJ	7 wk, F	1.7	6.4	7.6
BAlb/CJ	7 wk, F	1.7	6.4	7.6
129/J	7 wk, F	1.7	6.2	7.6

Although preliminary, these data suggest that males may be more resistant than females to lethal C. burnetii infection, and that age, at least in the limited range tested, had little effect.

The screening studies were extended to include outbred hamsters (LaK:LVG(SYR), 5 strains of inbred hamsters (MHA/Ss, LSH/Sx, CB/Ss, PD4/LAK, and LHC/LaK) all from Jackson Laboratory, as were outbred (TUM:MON) and inbred (MON/TUM) mongolian gerbils provided by Tumblebrook Farms, Inc. Animals of both species were 7 to 8 weeks of age, and as with the mice, were inoculated i.p. with $10^{6.5}$ $GRIPID_{50}$ of C. burnetii in phase I.

The infection was apparent in hamsters; all remained healthy and vigorous throughout the 4 week post-inoculation period. By contrast, 1 of 5 inbred and all (4 of 4) outbred gerbils died within 7 to 11 days after inoculation. A more extensive test currently in progress will provide preliminary histopathological data from infected gerbils, and determine the LD_{50} dose of C. burnetii for these potentially useful rodents.

Little is known about genetic control of resistance of mice to lethal C. burnetii infections. Screening studies suggest that mice do not differ dramatically in susceptibility. Most are resistant, suggesting that resistance may be controlled by one or more dominant genes. However, in search of a susceptible mouse, we have cross bred several of the more sensitive inbred strains that were identified during the screening study. First generation progeny from each of these crosses are currently being tested for susceptibility. Subsequent tests will be conducted after these recombinant inbred strains have reached the F4 generation of inbreeding.

The A/J strain mice appear to be the most sensitive to Q fever of all inbred mice tested to date. The ability of this strain to mount a protecting immune response is currently being examined. Mice were given 2 s.c. injections, 7 days apart, of inactivated phase I C. burnetii. The humoral antibody response, based on both IFA and ELISA assays, of the vaccinated mice will be followed for a period of 12 weeks after vaccination. After 8 weeks, groups of vaccinated and control mice will be challenged with $10^{7.0}$ $GPIPID_{50}$ of virulent C. burnetii and observed for illness and death.

While these animal experiments are progressing, efforts are underway to adapt sensitive enzyme-linked immunosorbent (ELISA) procedures for titrating antibodies against C. burnetii and for detecting C. burnetii antigens in infected animal tissue. Methods originally described by Engvall and Perlmann (23) and adapted by Yolken (24) were used with minor modifications. Three antigen preparations, all derived from the 3rd and 4th passage of the rickettsia purified from infected yolk sacs of embryonated chicken eggs, have been successfully used for titrating anti-C. burnetii antibodies by these procedures; 1) Q fever vaccine, phase I, Code No. NDBR 105 containing 3×10^9 cells per ml; 2) Soluble tricholoracetic acid extract of the phase I antigen component, and 3) the phase II residual cells remaining after TCA extraction of phase I components. Particulate antigens were settled by centrifugation and bound in the wells of polystyrene Micro ELISA plates that had been pre-treated with ammonium sulfate fractionated goat anti-C. burnetii antibodies. Trapping antibody was not needed to bind soluble antigen in wells of polyvinyl chloride plates. Heat inactivated serum samples were added to wells containing bound antigen and incubated for 2 h at 37°C. After washing the wells, species specific anti-IgG conjugated with alkaline phosphatase was added,

followed by another incubation interval and washing step. All intermediate wash steps of wells were done 5 times with 300 l of phosphate buffered saline containing 0.05% Tween-20. Alkaline phosphatase activity was measured by adding p-nitrophenyl phosphate and reading the absorbance at 405 m after 60 min incubation at 37°C.

With test samples of sera from infected mice and guinea pigs, the titration curves of antisera were linear over a wide range of serum dilutions, and end point titers were 5- to 20-fold higher than those obtained by IFA. Preliminary efforts have indicated that similar procedures, in which infected tissue is substituted for specific antigen, can detect 10^0 rickettsia per gram of infected mouse spleen. The greatest difficulties thus far encountered with these procedures are related to nonspecific reactions causing high background absorbance not due to the presence of specific antibody or antigen. Interactions among antisera, between antisera or enzyme-conjugated antibodies and the solid phase, or the presence of material in specimens which react nonspecifically with animal immunoglobulins may be the source of these difficulties. Preparation of more highly purified antigens (in progress), and antisera of the highest possible affinity, optimization of reagent concentrations and reaction times may reduce these nonspecific reactions and increase sensitivity.

As reported in the USAMRIID Annual Report 1981, an antiserum-containing agar medium was developed which permitted simultaneous detection and isolation of all Legionella species and serotypes of L. pneumophila known to us at the time of the report. A recently reported new species, L. longbeachae, has been obtained and included in the polyvalent vaccine used to produce antiserum in a goat and a rabbit, in order to have an adequate supply of up-to-date polyvalent antiserum agar to apply to clinical and epidemiological problems as they arise. Two practical problems with the antiserum agar method have been detected. First, pooled polyvalent rabbit antiserum stored at -10°C for three months did not produce precipitin rings around colonies of Legionella organisms growing on antiserum agar, and when titrated by the double diffusion agar gel precipitin method (Ouchterlony) had no detectable titer against any Legionella species or L. pneumophila serotypes; with the exception of serotype 3 antiserum which had a titer of 1:32, as when first obtained. Second, precipitin rings will only develop around well isolated Legionella colonies growing on antiserum agar, and some batches of the growth medium, yeast extract agar, did not permit growth of individual colonies although confluent growth did occur.

Phenotypic differences between the Pontiac Fever and Legionnaires' Disease isolates. Legionnaires' Disease and Pontiac Fever are markedly different clinical diseases in humans, yet the agents which caused the classic Legionnaires' Disease outbreak in Philadelphia and Pontiac Fever outbreak in Pontiac, Michigan, are reported to be genotypically and phenotypically indentical to L. pneumophila serotype 1. Although Legionnaires' Disease appears to have a low attack rate and rarely infects healthy young adults, Pontiac Fever outbreaks indicate that the causative agent is highly infectious and causes severe incapacitating disease in individuals of all ages; therefore a method for distinguishing between the Pontiac Fever and Legionnaires' Disease agents could be very useful in assessing their potential threat to military personnel. Differences in cultural characteristics between the Pontiac isolate and other L. pneumophila isolates were noted by us in

1978, therefore a study comparing the cultural characteristics of the Portiac Fever isolates with prototype isolates of all known Legionella species grown on four different media was conducted. No significant differences could be detected over 30 days incubation at 35°C and 80% relative humidity.

Effect of Legionella infection and intoxication on host phagocyte antibacterial activity. A published method developed in our laboratory for determining the antibacterial activity of mouse peritoneal phagocytes in vivo (1) has been used in an attempt to detect antiphagocytic effects of L. pneumophila infection and toxin on guinea pig caseinate-induced peritoneal exudate phagocytes in an in vitro system. No evidence could be detected that ingestion and killing of standard test organisms (Vibrio parahemolyticus) by guinea pig peritoneal exudate phagocytes was affected by infection with L. pneumophila organisms, nor by contact with Legionella toxin or anthrax toxin under the conditions of this assay. However, the guinea pig peritoneal exudate phagocytes did not undergo the morphologic changes observed in mouse peritoneal phagocytes when suspended in the hypertonic medium required for growth of phagocytosed test organisms, so that failure of phagocytosed organisms to grow may have been due to impermeability of guinea pig phagocytes to the medium rather than a bacteriocidal effect of phagocytosis.

Research with Drs. Lowry, Berendt, McNamee, and Hammond involves studies in the pathophysiology of aerosolized infectious bacterial agents using Legionella pneumophila. Two runs of guinea pigs exposed to a planned aerosolized LD_{50} dose of Legionella pneumophila were disappointing as the team was unable to consistently culture virulent organisms from solution for baseline data, or from tissue. Work with a CDC culture obtained in July tends to implicate technical problems rather than loss in pathogenicity. No further aerosolization is anticipated until this problem is resolved.

The surgical technique to bisect the trachea of guinea pigs has been perfected to a 5 minute operation, but preservation of the integrity of the tiny trachael lumen is a real problem: 1) anything causing sloughing of the trachael mucosa (aspiration by even soft polyethylene, etc.) leads to eventual luminal blockage and respiratory distress, 2) "fixing" of the skin-wound by collodian, crazy glue, nail polish, etc., is necessary to prevent oozing of blood and serum which runs into the trachael lumen when the animal becomes active, 3) tiny drops of blood and/or serum from the raw trachael operative surface escape into the lumen and coalesce on the mucosal lining to compromise the airway. Hemostasis, including miniscule oozing, must be completely achieved before the animal leaves the surgical table. Animals whose trachael stump was immediately painted with a solution of thrombin lived 5 days, and appeared "normal". The surgeon is pursuing this course.

A list of all USAMRIID personnel who have worked with Legionella pneumophila has been furnished to COL James B. Hammond to ascertain "before and after" serum titers or lack thereof. Cultures of water faucets, shower heads, water lines, etc. in the suite are being carried out for Legionella. Hospitals culturing Legionella from water and water lines report these as possible sources of their epidemics (25, 26, 27).

Finally the last research work with Dr. Knudson, Captains Mikesell and Dreier deals with the role of extrachromosomal elements that affect pathogens of military importance. Basically this research effort involved several different approaches within the same group as well as collaborative efforts with Dr. Vodkin of the Pathology Division. The major findings thus far have been the identification of a plasmid within B. anthracis that controls the production of toxin (PA, LF, and EF). Whether or not this plasmid contains the structural or merely regulatory genetic elements for toxin components is still unknown. Various strains of B. anthracis have been cured of their plasmids either by using elevated growth temperature or with novobiocin and these cured strains have been demonstrated to no longer be able to produce protective antigen, lethal factor or edema factor (see publication #6).

Studies on DNA repair. The ability to recover from injuries, whether mechanical, chemical or radiation, is characteristic of all living things and favored by natural selection. Therefore, it is not surprising to find genetic systems which enable bacteria and their viruses to recover from the potentially lethal effects of ultraviolet light, nor is it surprising to find these repair systems tightly regulated and subject to loss by mutation (4, 5). Dr. Knudson has recently written a definitive review of the role of inducible DNA repair in W-reactivation and related phenomena which will be published in Volume 8 of Progress in Molecular and Subcellular Biology. By the conjugal transfer of plasmids pKM101 and R68.45 into Serratia marcescens, Dr. Knudson demonstrated plasmid mediated resistance to killing by UV-irradiation (11). Studies are presently underway to determine the UV-repair capabilities of B. anthracis and Legionella species and to elucidate the role of plasmids in these repair processes.

Isolation of plasmids from B. anthracis. The first step was to screen and compare vaccine strains and wild type strains of B. anthracis for extrachromosomal DNA and to characterize these plasmids in terms of molecular weight. A computer literature search revealed that there was no published reports of plasmids in B. anthracis. Dr. Knudson developed a simple and rapid plasmid isolation procedure for B. anthracis which gives distinct plasmid bands with agarose gel electrophoresis. As little as 0.05 g of DNA can be detected. This procedure, which is modified from a method by Kado and Liu (28), releases DNA from B. anthracis by using alkaline sodium dodecylsulfate (pH 12.6) at elevated temperatures. Protein and cell debris are removed by extraction with phenolchloroform. The clarified extract is used directly for electrophoretic analysis. For CsCl-EtBr gradient ultracentrifugation, Sarkosyl was substituted for SDS. Using this plasmid isolation procedure, Dr. Knudson has isolated plasmids from several laboratory and wild type isolates of B. anthracis including Sterne, V770, V770-NP1-R, 107-NP2-R1, Vollum, and from primary isolates obtained from Iowa, Colorado, and Texas. Using agarose gel electrophoresis of DNA isolated from these strains of B. anthracis., Dr. Knudson demonstrated that Sterne and V770 contain a large molecular weight plasmid and a small molecular weight plasmid while the encapsulated virulent strains contain an additional large molecular weight plasmid.

Molecular weight determinations. Dr. Knudson has purified the plasmid DNA by CsCl-ethidium bromide density gradient ultracentrifugation. The lower band in these gradients was plasmid DNA while the upper band contained chromosomal DNA. The bands were removed from the gradient, partitioned with isopropanol to remove the EtBr and dialysed to remove the CsCl. This DNA was then electrophoresed on a 0.7% agarose gel and stained with EtBr. A new set of plasmid standards for molecular weight determination by agarose gel electrophoresis was obtained from Dr. Esther Lederberg,

Plasmid Reference Center, Stanford University. These E. coli strains contain the following plasmids: R40a (MW = 96 X 10^6); R62 (MW = 80 X 10^6); R1 (MW = 60 X 10^6); and RP4 (MW = 34 X 10^6). The molecular weight of the plasmid DNA isolated from the Sterne strain of B. anthracis was estimated from its relative mobility on agarose gels compared to the relative mobility of the reference plasmids of known molecular weight. There is a linear relationship between the log of the molecular weight of the plasmid and the log of the relative mobility. The molecular weights of the plasmid DNA from the Sterne strain was determined by Dr. Knudson to be approximately 95 Mdal and 20 Mdal, using a least squares regression plot. The molecular weight was also estimated to be about 95 Mdal by taking the sum of the molecular weights of the plasmid restriction fragments.

Generation of plasmid-free isolates. Curing, which results in plasmid-free cells, was accomplishd by inhibiting the replication of the autonomous plasmid DNA while allowing the bacterial chromosomal DNA to continue to replicate. Both plasmid and chromosomal replication are hindered by acridines but the necessity for growth during curing selects cells in which chromosomal replication, but not necessarily plasmid replication, has been achieved. The effective curing concentration, which is just less than that inhibiting growth, was established for acridine orange, ethidium bromide, and novobiocin. The minimal inhibitory concentrations were considerably less for B. anthracis (Sterne) than for E. coli. Elevated temperature was also used for curing since some plasmids are inhibited at 42°C. Since there were no known markers on the plasmids in B. anthracis, individual colonies had to be tested separately on agarose gels following treatment with curing agents. In this way Dr. Knudson isolated the first strains of B. anthracis cured of the large molecular weight plasmid.

Plasmid-associated toxin production in B. anthracis. B. anthracis (Sterne) cured with novobiocin at 42°C and nontreated control cells were grown to 1 X 10^8 cfu/ml in a synthetic medium which optimizes toxin production. Dr. Knudson then demonstrated that an i.v. injection of 2 ml of culture filtrate from nontreated, plasmid containing Sterne strain killed Fisher 344 albino rats with an average time to death of 60 min whereas two, four, six, and eight ml of culture filtrate from the cured Sterne strain, which lacks the large molecular weight plasmid, did not kill when given i.v. This clearly demonstrates the association of the large plasmid with toxin production in B. anthracis.

Screen for other plasmid-associated metabolic marker. Dr. Knudson examined V770, Sterne, and a cured Sterne isolate (GK8N) for other biochemical differences. Each strain was tested for drug resistance by the Kirby-Bauer method using 16 different antibiotics. There were distinct differences between V770 and Sterne in their resistance to methicillin and erythromycin. Sterne and the cured Sterne (GK8N) strains showed slight differences in resistance to sulfisoxazole. The strains were also tested for differences in 35 metabolic markers. V770 and Sterne showed distinct differences in gelatin hydrolysis. Dr. Curtis Thorne demonstrated that B. anthracis produces a bacteriocin against B. thuringiensis and confirmed that it is not plasmid linked.

Immunodiffusion assay. Novobiocin cured strains and wild type strains were transferred with sterile toothpicks to plates of chemically-defined medium containing antiserum from goats that had been inoculated with B. anthracis (Sterne) spores. Following a 24 h incubation in a CO_2 incubator, halos of precipitate surrounded untreated plasmid containing colonies while halos were not seen around plasmid free novobiocin treated isolates.

Cloning the protective antigen gene from B. anthracis. Dr. Knudson began this study of B. anthracis genetics by looking for plasmids and their involvement in toxin production. There were several reasons for suspected plasmid involvement. Anthrax is a toxigenic disease and toxin production is plasmid-mediated in many other pathogens. Furthermore, several species of Bacillus are known to contain plasmids.

There is a pressing need for a more effective anthrax vaccine. Protective antigen, which is one of the B. anthracis toxin components, is the substance in the presently used crude vaccine which is primarily responsible for the development of immunity. Protective antigen, which has been shown to be an effective immunogen for protection against a spore challenge, could be produced in large quantities by cloning the gene for protective antigen. Since Dr. Knudson has shown that the B. anthracis toxin production is plasmid-associated, his efforts have been concentrated on cloning the B. anthracis plasmid restriction fragments.

The first step was the isolation and purification of the B. anthracis plasmid DNA by CsCl-EtBr ultracentrifugation of Sarkosyl-lysates. Dr. Knudson has recently increased the purity of the B. anthracis plasmid DNA from SDS- lysates by extracting it from the agarose electrophoretic bands using low melting point agarose followed by EtOH precipitation. This method is faster than CsCl-EtBr ultracentrifugation and results in a plasmid preparation with less contaminating chromosomal DNA. The second step was to cleave the B. anthracis plasmid DNA with various restriction enzymes, including Eco R1, Hind III, Pst I, and Bam, and then to separate the restriction fragments by electrophoresis. The vectors pRB322 and pRB325 were also linearized with restriction enzymes. The vectors were then treated with bacterial alkaline phosphatase (BAP) which removed the 5'-terminal phosphate groups thus preventing recircularization and plasmid dimer formation. The third step was ligation. Pst I restricted B. anthracis plasmid DNA was mixed with BAP treated vector DNA along with DNA ligase. Ligations were also run with Eco R1 cut pBR325 (with and without BAP) and Eco R1 E. coli DNA restriction fragments. Self ligation of Eco R1 cut plasmids, without BAP treatment, was demonstrated by gel electrophoresis. The next step was transformation. Competent cells in $CaCl_2$ were transformed with pBR322 and screened for resistance to ampicillin and tetracycline. Transformation with uncut pBR322, uncut pBR325, and with pBR322 linearized with Pst I and religated with T4 DNA ligase, all resulted in expected levels of transformed cells. Transformation with vector DNA ligated with B. anthracis plasmid fragments gave very low yields.

The final step is screening. Novobiocin cured strains and wild type strains of B. anthracis were transferred with sterile toothpicks to plates of chemically-defined medium containing antiserum from goats that had been inoculated with B. anthracis (Sterne) spore. Following a 24 h incubation in a CO_2 incubator, halos of precipitate surrounded untreated plasmid containing colonies while halos were not seen around plasmid free novobiocin treated isolates. This immunodiffusion assay will be used to screen the E. coli transformants for B. anthracis toxin production.

Presentations:

1. Wachter, R. F. and G. P. Briggs. Isolation of phase I antigen from trichloroacetic acid (TCA) extracts of Coxiella burnetii. Presented, 82nd Annual Meeting of the American Society of Microbiology, Atlanta, GA, March 1982.

2. Lowry, B. S., R. F. Berendt, and G. A. McNamee. Guinea pig respiratory tract invasion following aerosolization by Legionella pneumophila. Presented, American Society of Clinical Pathologists, New Orleans, LA, March 1982 (abstract 78:264).

3. Lowry, B. S. Research in Legionella pneumophila. Presented, Junior Board, American Lung Association, Frederick, MD, March 1982.

4. Knudson, Gregory B. Genetic engineering and the control of human heredity. Invited address, Myersville Lions Club, Frederick, MD, November 1981.

5. Knudson, G. B., B. Ivins, and P. Mikesell. Plasmid-associated toxin production in Bacillus anthracis. Presented, 82nd Annual Meeting of the American Society for Microbiology, Atlanta, GA, March 1982.

6. Mikesell, P., T. Dreier, J. D. Ristroph, and G. B. Knudson. Plasmid isolation in virulent and avirulent strains of Bacillus anthracis. Presented, 82nd Annual Meeting of the American Society for Microbiology, Atlanta, GA, March 1982.

7. Knudson, G. B. Genetic engineering: The ultimate technology. Invited address, University of Maryland Dental School, Baltimore, MD, April 1982.

8 Knudson, G. B. Bacillus anthracis: An historical perspective. Invited address, Walter Reed Army Institute of Research, Washington, D.C., June 1982.

9. Mikesell, P. and G. B. Knudson. Plasmids of Legionella species. Presented, Army Science Conference, West Point, NY, May 1982.

10. Mikesell, P., B. E. Ivins, and J. D. Ristroph. Evidence for plasmid-mediated toxin production in Bacillus anthracis. Presented, FASEB Annual Meeting, New Orleans, LA, April 1982.

11. Knudson, G. B. and F. A. Galiano. Photoreactivation in Bacillus anthracis. Presented, 83rd Annual Meeting of the American Society for Microbiology, New Orleans, LA, 6-11 March 1983.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DAOG 3854	82 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM
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10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AP	198
b. CONTRIBUTING	61101A	3A161101A91C	00	
c. CONTRIBUTING	STOG 80-7.2:	2		

11. TITLE *(Precede with Security Classification Code)*

(U) Biology of Viral Agents of Potential BW Importance

12. SUBJECT AREAS

003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	12.9	775
c. TYPE	d. AMOUNT	83	11.0	675
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS *(include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR Dalrymple, J M
d. TELEPHONE NUMBER *(include area code)* 301-663-2833	d. TELEPHONE NUMBER *(include area code)* 301-662-7241
21. GENERAL USE FIC MILITARY/CIVILIAN APPLICATION:	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* White, J D g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Viral Diseases; (U) Prophylaxis; (U) Laboratory Animals; (U) Chemotherapy; (U) Vaccines

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Elucidate replicative mechanisms and antigenic composition of selected toga- and bunyaviruses that are unique in their capacity to cause widespread epidemics of military significance. Physical and biochemical characterization of viral constituents allow the identification of immunogens of potential prophylactic value. Clearly defined virus-specific replication mechanisms will be investigated as targets for antiviral chemotherapy and clues to pathogenicity, to provide the scientific base for vaccine and drug development to protect military personnel.

24. (U) Characterize the structural protein antigens of selected pathogens using biochemical and biophysical separation methods, as well as define specific antigens and functions using specific antibodies. Characterize nucleic acids and deduce the replication strategy of representative alpha-, flavi-, and phlebovirus pathogens in an attempt to evaluate various methods for disease control.

25. (U) 8110 - 8209 - Hantaan virus, the etiologic agent of Korean hemorrhagic fever, has been propagated to high titer in cell culture and a sensitive plaque assay for detecting virus infectivity and neutralizing antibody has been developed. The development of a sensitive radioimmune assay and methods for concentration and purification allowed preliminary characterization of the virion. Hantaan virus contains 3 RNA species and at least 2 unique structural proteins. Clones of DNA have been prepared to the M-segment RNA of Rift Valley fever (RVF) virus and used for hybridization experiments. Preliminary experiments with Junin virus have allowed partial characterization of the RNA genome. Lymphocyte hybridomas producing monoclonal antibodies to RVF and Japanese encephalitis virus have been produced. Publications: Chapter in The Replication of Negative Strand Viruses, 1981. These studies were continued from 3A161101A91C-143, Accession No. DAOG 3854.

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)

Work Unit No. S10-AP-198: Biology of Viral Agents of Potential BW Importance

Background:

During the period of this report, considerable progress was made on the characterization of Hantaan virus (HTNV), the etiologic agent of Korean Hemorrhagic Fever (KHF). Following the development of sensitive assays for virus infectivity (plaque assay), virus antigen (radioimmune assay and ELISA) and antibody (plaque reduction neutralization test), methods and procedures were described for optimal propagation, concentration, and purification of cell adapted HTNV. Spherical to oval particles with a unit membrane and subunit surface structure were demonstrated by negative contrast staining. Purified virions were shown to contain three separate and unique RNA species and at least two resolvable proteins. RNA extracted from partially purified HTNV was visualized by electron microscopy and immunospecific labeling of smooth membraned vesicles was demonstrated in virus infected cells in the absence of recognizable intracellular virions.

Research on virus antigen characterization continued using primarily monoclonal antibodies. During this reporting period, lymphocyte hybridomas producing monoclonal antibodies to Punta Toro and Rift Valley Fever (RVF) virus were prepared and characterization initiated. Although more hybridomas producing different monoclonal antibodies to these viruses will be required to provide complete descriptions of the antigenic determinants available, many of these reagents have already proven useful in rapid viral diagnosis and differentiating virus strains.

A continuing research effort directed toward the expression of an immunogen of potential prophylactic value against RVF virus infection using recombinant DNA technology required the close collaboration of in-house research efforts and the staff of Molecular Genetics Inc. (MGI). Multiple preparations of the m-segment RNA from RVF virus were purified, safety tested and sent to MGI laboratories. A c-DNA clone was used to develop procedures for propagation, isolation and characterization of DNA clones from plasmid vectors and host bacteria. Hybridization studies have shown this clone to be an extremely useful probe for the detection and ientification of RVF virus m-segment RNA.

Studies on the evaluation of oligonucleotide mapping as a technique for estimating homology between dengue-1 strains were completed and preliminary characterization of Junn virus RNA was initiated. This change in research emphasis reflects a shift in direction toward the characterization of the more hazardous agents being researched at USAMRIID using techniques developed with agents requiring lesser containment.

Progress:

HTNV is the name given to the virus registered as the etiologic agent of Korean Hemorrhagic Fever (KHF) or hemorrhagic fever with renal syndrome. The prototype virus was originally isolated from the Korean striped field mouse, Apodemus agrarius coriae, and this strain (#76-118) was passaged five times in sero-negative mice by

injection of antigen positive lung material. HTNV (#76-118) was successfully adapted to cell culture at USAMRIID by French and co-workers by infecting human alvelor carcinoma cell line with infectious mouse lung material. Although many of the basic characteristics of the virus have been described, taxonomic placement and molecular chacterization have awaited the development of the in vitro technologies necessary for propagating and assaying high titered virus suspensions. Development of such methods and partial characterization of the agent are the subject of this report.

A variety of HTNV isolates have been examined at USAMRIID, however, the #76-118 strain remains as the only isolate well adapted for replication in cell culture. Many of these other strains have exhibited evidence of contamination with Reovirus-like agents. For these reasons, the #76-118 strain of HTNV has been adopted as the virus strain used for all subsequent characterization work and other strains with suspected contamination have been confined to an isolated laboratory in which elimination of the contaminants is being attempted.

Early studies on virus characterization were performed using virus that had been passaged 15 times in A549 cells. Attempts to propagate this virus in a clone of Vero cells (Vero E-6) revealed that virus titers were lower than comparable studies in A549 cells. A new preparation of virus that has been passaged only three times in A549 cells and two additional passages in the Vero E-6 cells currently serves as seed virus. This virus preparation results in higher yields of infectious virus from Vero E-6 cells.

The passage level of virus used as infective inocula appeared important in obtaining high titered virus yields. The first passage in Vero E-6 cells (A549 passage 3 inoculum) yielded only 10^4 PFU/ml maximum titer and this did not occur until 10-12 days post-infection. A second passage in Vero E-6 cells resulted in a maximum titer of greater than 10^7 PFU/ml at about 7-9 days post-infection. A third passage in Vero E-6, using undiluted 10^7 PFU/ml virus from the second passage, resulted in a maximum titer of less than 10^6 PFU/ml, but the maximum titer was obtained at approximately 2 days post-infection. Current procedures involve the infection of Vero E-6 cells with a second passage virus diluted 1:500-1:1000 and harvest of infectious supernatant fluids 7-10 days post-infection. This method consistently yielded virus preparations titering in excess of 10^7 PFU/ml. The presence of defective interfering particles or some other interfering substance(s) in the infecting virus inoculum cannot be extended and is currently under investigation.

A plaque assay for HTNV has recently been developed for cell cultute adapted HTNV based on the previous work by Dr. Joseph McCormick and colleagues at CDC, Atlanta. Of some 20 different cell lines examined, only 3 lines supported the plaquing of HTNV, Vero E-6, A549, and LLC-MK_2. Of these, the Vero E-6 cell must be considered the superior substrate because of the clarity of the plaques and the ability of these monolayers to withstand long incubation times under agarose overlays. It was interesting to note that the Vero cells were negative in plaque assays even though they were the parent cells from which the E-6 clone was derived. All other cells tested were negative in HTNV plaque assays, even with numerous changes in the composition of the overlay such as the addition of heparin, dimethyl sulfoxide, tryspin, and/or DEAE dextran. It should be mentioned that other cell lines could support the replication of HTNV even though they were an unsatisfactory substrate for the plaque assay.

The plaque assay for HTNV, as originally described, required a full 14-17 days incubation before readily discernible plaques could be quantitated. Using the current assay with slight modifications, such as incubation at 38°C and virus passaged through Vero E-6 cells, culture can be stained as early as day 5 or 6 and plaques counted on day 6 or 7. Large, medium, and small plaques could be distinguished and purified preparations of each plaque-size virus could have been prepared by plaque selection and subsequent plaque-to-plaque passage. Each of the plaque preparations have been verified as HTVN by neutralization studies using KHF convalescent sera.

Plaque reduction neutralization tests have been performed on a battery of acute and convalescent KHF sera from a variety of sources. Both large and small plaque viruses were neutralized in the presence of antibody and no non-neutralizable fraction of virus could be detected. The addition of fresh non-immune serum (2% monkey serum) to the virus antibody mixtures appeared to significantlyu increase the 80% plaque reduction neutralization endpoint titer. Although complement sources such as guinea pig serum were also shown to increase neutralization titers, they were not as effective as fresh monkey sera. Using "assessory factor" or fresh monkey sera human serum neutralization antibody titers approached indirect fluorescent titers. This was true for all convalescent KHF sera from Korea, but fluorescent antibody titers on Nephropathia epidemica sera from Scandinavian cases were still considerably higher than neutralization titers. These data suggest that the neutralization test can be used to confirm fluorescent antibody serology, but probably is quite specific for HTNV strains from Korea and surrounding areas.

Virus concentration and purification studies have been previously hampered by the relatively poor virus yields from infected cell cultures and the apparent instability of the virus particle during most purification and analytical procedures. Using virus pelleted in the ultracentrifuge or concentrated by polyethylene glycol precipitation and sedimentation on rate zonal sucrose gradients for short centrifugation times, we have been able to demonstrate the coincident sedimentation of virus infectivity measured in the plaque assay and virus antigen detected by simplified radioimmune assay. (RIA). The RIA employed involves the electrostatic attachment of antigen to a polyvinyl chloride microtiter plate, the addition of convalescent human or immune experimental animal antisera, and the detection of bound immune complexes with iodinated protein A. Using this assay, multiple sedimenting antigenic species can be detected in concentrated virus preparations.

HTVN has been reported as an RNA virus based primarily on the insensitivity of virus replication to actinomycin D. To more clearly define this RNA structure, virus was radiolabeled with ^{32}P from 6-9 days post-infection in Vero E-6 cells. Ultracentrifuge pelleted material was sedimented to near density on sucrose gradients and peak virus antigen fractions detected by RIA. Although precise density measurements in other gradient media have not been completed, the major peak of sedimetable antigen appeared at a density of 1.2. Nucleic acid extracted from this peak and subjected to electrophoresis on agarose gels revealed three distinct and well-separated RNA species by autoradiography.

Although only preliminary molecular weight estimates have been made, using 28S and 18S host cell ribosomal RNAs as markers, a value of 2.6 million, 1.2 million, and 0.6 million daltons have been tentatively assigned to the L, M, and S segments, respectively. All three species are sensitive to ribonuclease digestion and the equidistant separation of the three RNAs on agarose gels is quite different from the

migration of Rift Valley Fever virus. Preliminary data from oligonucleotide maps of T_1 ribonuclease digests of each of the RNA segments clearly showed these RNA species to be unique to HTNV and separate from the cellular ribosomal RNAs or segmented viruses currently bring investigated in the laboratory.

Virus proteins were not emphasized in early experiments because of the chronic nature of the infection and our inability to reduce the uptake ot radioactive protein precursors into normal host cell proteins. In preliminary experiments using concentrated and purified ^{35}S-methionine radiolabeled HTVN, only a single polypeptide of 50-55,000 daltons could be resolved on discontinuous polyacrylamide gels. A polypeptide in the 50-55,000 MW range has also been detected in both purified virus and infected cell lysates using immunodetection of antigen from polyacrylamide gels blotted onto nitrocellulose paper. More recently, a second structural polypeptide of approximately 65,000 daltons has been detected in preparations of purified radiolabeled virus. The detection of only two proteins probably reflects the low yields of purified virus or the insensitivity of immunodetection methods since most viruses wiht similar characteristics contain three or more structural proteins.

The increased yields from infected cells, sensitive methods for detecting infectious virus and antigens and methods for concentrating and purifying HTNV, have contributed significantly to the characterization of the virus. Progress from thsi point should be much more rapid and complete biochemical characterization of HTNV now appears feasible.

Supernatant fluids from A-549 cell cultures infected with HTNV inocula in the 9th to 15th passage contain round to oval particles which vary in diameter from 80 to 110 nm (average, 95 nm). Particles have a regularly spaced subunit structure which is revealed by glutaraldehyde treatment and consists of surface projections.

To determine whether or not the observed particles were associated with virus activity, isolation of the virus was attempted in sucrose gradients. Maximum virus infectivity was located at buoyant densities of 1.15 to 1.18 g/ml in repeated experiments. Virions were found by electron microscopy only in fractions at or adjacent to those with maximum infectivity.

These data suggested that the morphology of HTNV is Bunyaviridae-like (1,2) which is consistent with published pysicochemical properties (3,4) and the results of this report describintg three bands in agarose gels of RNA extracts from HTNV.

IN pursuit of additional supportive information, we attempted to visulize genome segments of HTNV by electron microscopy. Supernatant fluids from infected A-549 cell cultures containing 3.0×10^6 fluorescent focus foroming units (FFU) were clarified by filtration and stirred overnight in 10% polyethelyne glycol (PEG) 6000 and 0.5M NaCl. Approximately 46 percent of the original infectivity was recovered in 18-fold concentration after the precipitate obtained by centrifugation at 10,000 X g was dissolved in TNE buffer. A 5 ml volume was applied and centrifuged on a reorienting linear sucrose gradient; however, the seal leaked on the tube containing th virus gradient during centrifugation. This disturbed the linearity of sucrose densities in the gradient during the reorientation phases and assay of 2 ml fractions showed nearly equal FFU titers throughout all fractions. All the fractions were pooled, diluted with 9 volumes of TNE buffer and treated with PEG as above. The precipitate was dissolved in TNE buffer, layered over 15 percent

ribonuclease-free sucrose and centrifuged at 132,000 g for 1.5 hr. This precipitate was desolved in TNE buffer and the RNA, extracted with sodium dodecyl sulfate and phenol, was prepared for electron microscopy (6). Total RNA was examined without fractionation and lengths were determined after spreading on formamide. The results are shown in Table I. All molecules were linear strands; no circular strands were seen. Statistical analysis of 401 molecules indicates a minimum of two size diseases. Approximately 7.5 percent of the molecules were markedly longer with an average length of 1.33 nm. The length of the other molecules varied from 0.2 to 0.9 m. A peak frequency at 0.35 nm appears skewed indicating a high background (i.e., fragments of larger RNA molecules or cellular RNA) and/or the presence of an intermediate size class of RNA molecules with an average length greater than 0.5 nm.

A molecular standard was not included in this preliminary evaluataion of a technique for visualizing RNA molecules. Although we cannot estimate molecular weight, the frequency and distribution of 0.35- and 1.33-nm molecules is comparable to that described for Uukuniemi virus (5).

Cultures of A-549 cells have been used for studies of HTNV morphogenesis. Infected cell cultures do not display cytopathogenic effects and the ultrastructural morphology of these cells is virtually indistinguishable from normal ones. These observations are consistent for cultures in which 90 percent of the cells contain immunofluorescent (IF) antigen specifically detected with human convalescent serum. Occasionally, individual particles or clusters were seen near golgi-lamellae in otherwise normal appearing cells. These particles are 90-100 nm in diameter. Using procedures that permeabilize cytoplasmic membranes to antibody molecules without destroying the ultrastructure, we have attempted to detect particles by immunoelectron microscopy with ferritin (6). HTNV antigen was localized with rabbit antiserum to the heterologous of HTNV (1). The reaction product was concentrated within smooth membraned vesicles of HTNV-infected cell but no virus particles were seen.

The E-6 cell culture, a Vero cell clone, has been used to plaque assay HTNV[2,5]. We examined infected E-6 cultures in the electron microscope and compared the growth of A549 cell adapted HTNV in E-6 and A-549 cell cultures. Both cells are similar with respect to lack of ultrastructural changes after HTNV infection. Growth and development of intracellular antigen were evaluated in 6-well plates and 8-chambered slide cultures (LAB TEK^{R}, Miles Laboratories) by assay of supernatant culture fluid for PFU and immunofluorescent staining, respectively. Maximum infectivity titer appeared earlier in A-549 cells and was maintained at higher levels than in E-6 cells (Table II). The number of cells containing IF antigen and the time of its appearance in cells paralleled the infectivity titers with both cell lines.

Rift Valley Fever (RVF) virus remains an important human and animal pathogen and a disease threat of potential significance to the military. Virus research directed toward combating such a disease problem must include efforts to obtain rapid and sensitive virus and virus antibody detection systems as well as attempts to enhance human immunity through the development of improved vaccines. This research project has specifically applied two of the more recently developed tedchnologies to the study of the RVF problem, namely: lymphocyte hybridoma production of monoclonal antibodies and the production of specific immunogens using recombinant DNA. RVF virus has not been intensively studied in the U.S. primarily because of the requirement for containment; therefore considerable basic molecular characterization of the virus was a necessary prerequisite to these studies.

Following the development of methodologies neccessary for characterizing RVF virus, the virion structural proteins were isolated and identified. Lymphocyte hybridomas were prepared using standard procedures and two separate fusion experiments yielded a battery of monoclonal antibodies directed against RVF virus. The first fusion resulted in 13 hybridoma cultures that are currently frozen in the USAMRIID repository and the second fusion experiment resulted in an additional 36 cultures producing monoclonal antibodies. Although cultures from the latter expoeriments are similarly frozen at USAMRIID, they have not yet been passaged or further cloned and as a result, the immortality and future viability of each of these hybridomas cannot be predicted. In addition to the RVF virus experiments, two separate fusion experiments are performed using the closely related Phlebovirus, Punta Toro. Characterization of the monoclonal antibodies resulting from these fusions is being performed in collaboration with Dr. Jonathan Smith, a USAMRDC contractor at the University of Maryland Medical School, Baltimore, Maryland.

Since the solid phase radioimmune assay (SPRIA) was the assay employed for detecting antibody positive hybridoma cultures, all monoclonal antibodies react by SPRIA but could also be detected using ELISA or indirect fluorescent antibody procedures. Of those examined, none of the RVF virus monoclonal antibodies have been shown to inhibit viral hemagglutination or neutralize virus in plaque reduction neutralization tests. Using radioimmune precipitation and analysis of the hprecipitated virion components by polyacrylamide gel electrophoresis (PAGE), the majority of the monoclonal antibodies appeared to be directed toward the nucleocapsid protein of the virus. This would explain why hemagglutination inhibition and neutralization were not observed since the nucleocapsid is internal and does not participate in either of these viral functions. Monoclonal antibodies directed against the viral glycoproteins have been detected but precise identification of the specificity (G_1 of G_2) must await refinement of the raidioimmune precipitation test. The G_1 and G_2 envelope glycoproteins of RVF virus migrate very similarly on PAGE and clear resolution of antibody precipitated position is extremely difficult. The envelope glycoproteins of Punta Toro virus are more easily resolved by PAGE and separate G_1 and G_2reactivities have been desribed among these monoclonal antibodies. In contrast to the RVF virus experience, both neutralizing and hemagglutination-inhibiting antibodieshave been observed among Punta Toro monoclonal antibodies. These antibody functions do not appear to segregate with glycoprotein specificity and were observed with both anti-G_1 and anti-G_2 monoclonal antibodies.

Several of the TVF virus monoclonal anitbodies have been submitted to the Rapid Virus Diagnosis group at USAMRIID for evaluation as both capture antibody for virus detection systems as well as type specific and cross reactive immune reagents for virus identification. Many of these reagents were useful in antigen capture ELISA tests (Dr. Robert Rosato, personal communication) and still others exhibited potential in differentiating RVF virus from othe cross-reacting Phleboviruses and identifying separate strains of RVF virus (Dr. C. J. Peters, personal communication).

After demonstrating the utility of the methods and feasibility for using monoclonal antibodies for lymphocyte hybridomas to characterize virus antigens, a lymphocyte hybridoma production support facility has been installed at USAMRIID. Technical staff have bee trained by a workshop conducted at USAMRIID and this facility now functions under the direction of Dr. Elizabeth Early, Virology Division.

Production of RVF virus immunogens using recombinant DNA technology was pursued with the assistance of a USAMRDC contract with Molecular Genetics, Inc. (MGI) Minneronka, MN. Preliminary charcterization studies performed at USAMRIID included the isolation and identification of three virion RNA species from purified RVF virus nucleocapsids. The apparent MWs of the L, M, and S RNA species were determined to be 2.8×10^6, 1.8×10^6, and 0.74×10^6 daltons and each exhibited a unique fingerprint pattern by oligonucleotide mapping.

Multiple preparations of RVF virus M segment RNA were propagated, purified, analyzed, safety tested and sent to MGI for further characterization and gene cloning experiments. All RNA preparations were safey tested by direct plaque assay and attempted propagation in cell culture as well as the injection of suckling mice and weanling hamsters. No residual infectivity could be detected in any of these RNA preparations.

Following a series of preliminary gene cloning experiments at MGI, a single clone (DNA) consisting of 250 base pair was obtained, inserted into a plasmid vector, propagated in bacteria, isolated purified and delivered to USAMRIID for further analysis. The clone possesses an open reading frame suggesting that it may represent a portion of the code for RVF virus envelope glycoprotein. The predicted amino acid sequence from the clone would suggest a peptide with both hydrophilic and hydrophobic regions, adding to the possibility that such a peptide will be antigenic and immunogenic.

The 250 base pair clone has been radiolabelled by nick translation and used as a probe in RNA hybridization studies. The cloned probe hybridized specifically with M segment RNA from purified RVf virus nucleocapsids. Using a CsCl gradient fractionation method designed to pellet and thereby separate messenger RNAs from virion RNAs within infected cells, multiple virus specific RNAs were obtained. The radiolabelled DNA from the clone clearly hybridized to an RNA species in the pellet that was slightly smaller than M segment RVF virus RNA. These studies suggest that the DNA clone reflects as portion of the gene coding for RVf virus envelope proteins. Attempts to synthesize the predicted peptide and test for immunogenicity are in progress.

The potential for success of this project is enhanced by the encouraging results obtained from a single cDNA lcone in these early experiments. In addition to the ultimate objective of producing RVF virus immunogens of prophylactic value using recombinant DNA technology, the utilization of cDNA material as molecular probes is a tremendous aisd in molecular charcterization studies. Attempts to obtain longer clones representing more of the gene are being intensified and recombinant DNA technology is being incorporated into the USAMRIID laboratory technology base.

Oligonucleotide Mapping of dengue-1 virus strains

A project initiated at WRAIR and designed to evaluate the utility of RNA fingerprinting or T_1 oligonucleotide mapping in virus strain comparison was successfully completed during the period of this report. A summary (Table III) clearly illustrates that numerous dengue-1 virus strains can be compared and related to geographic areas of virus isolation. These studies have demonstrated the utility of this method as an assist in defining the epidemiology of dengue infections. Plans to incorporate this capability into the battery of methodologies available for disease surveillance and identification are being pursued by attempting to take advantage of contract mechanisms.

Characteristics of in virus nucleic acids

Junin virus (JV), the etiologic agent of Argentine hemorrhagic fever, is a highly virulent member of the Tacaribe complex of Arenaviridae. This virus, as well as certian other arenaviruses (Lassa and Machupo) can cause severe hemorrhagic diseases in man, however, they also react in serological tests with other members of this virus group that cause no human disease or only very mild symptoms. Because of the difficulties associated with laboratory containment, little molecular characterization of virulent arenavirus strains has been attempted. Such characterizaion is important for determining the genome and protein (antigenic) relationships of virulent and avirulent for and the differences in rplication strategies these viruses must assume in order to cause the wide variation in pathogenesis seen in both acute and persistent infections. Unique containment facilities at USAMRIID have allowed us to begin analyses of several laboratory attenuated and field isolated strains of JV to determine nucleic acid characteristics.

In collaboration with Dr. Kelly McKee, ^{32}P-labeled JV (XJ44 strain) propagated in Vero cells was concentrated by ultracentriguation and purified by sequential centrifugation on 20-60% discontinuous sucrose and 15 to 40% continuous Renografin^{R} graedients. These procedures were experimentally determined to be superior to a variety of methods tested. Infectivity titers i excess of 10^8 PFU/ml coincident with maximum isotopic label were observed in the single optically defined virus band. As with other arenaviruses, electrophoretic analysis of heat-denatured JV RNAs on polyacrylamide or agarose gels revealed four distinct RNA species. Large (L, 31D) and small (S, 26S) viral RNAs (RvRNAs) were observed along with cellular 28Sand 18S ribosomal RNAs. Apparent molecular weights of the L and S vRNAs were determined to be 2.7×10^6 and 1.5×10^6 daltons, respectively. The L segment vRNA appeared to possess secondary structure based upon differences in electrophoretic migration observed under denaturing and non-denaturing conditions. Oligonucleotide fingerprint analysis of the two cRNAs confirmed their uniqueness as disinct vRNA species. There did not appear to be any overlap of the large oligonucleotides between the two vRNA species or between the vRNAs and celluar RNAs. Few, if any similarities were noted between fingerprint pattens of JV vRNas and those reported for other arenaviruses. Ongoing RNA and protein analyses of other JV strains (e.g. XJ13, Candid #1 vaccine strain) are in progress and should demonstrate the utiltiy on a biomedical basis. These studies will eventually encompass both Lassa and Machupo strains.

The biomedical and biophysical characterization of viral agenst of potential BW importance remains as a major objective of this research. The development and application of new technologies to the study of these agents should result in improved methods for the production of immunogens, detection of antigens, control of virus infection and lead to a more complete understanding of replicative mechanisms and possible intervention for disease control.

Presentations:

1. Rift Valley Fever Virus--RNA and Protein Characterization by P. Repik, B. J. Erlick, S. Harrison, and J. M. Dalrymple. 30th Annual Meeting of the American Society of Tropical Medicine and Hygiene, November 1981.

3. Oligonucleotide Markers of Attenuated Dengue-virus Vaccine Strains by P. Repid, K. H. Eckels, and J. M. Dalrymple. 1st Annual Meeting of the American Society for Virology, August 1982.

Publications:

1. White, J. D., F. G. Shirey, G. R. French, J. W. Huggins, O. M. Brand, and H. W. Lee. 1982. Hantaan virus, aetiologic agent of Korean hemorrhagic fever, has Bunyaviridae-like morphology. Lancet 1:768-771.6.

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TABLE 1. LENGTH OF DISTRIBUTION OF RNA MOLECULES

Length nm	Number of molecules
0.10 - 0.19	15
0.20 - 0.29	52
0.30 - 0.39	76
0.40 - 0.49	57
0.50 - 0.59	57
0.60 - 0.69	37
0.70 - 0.79	29
0.80 - 0.89	25
0.90 - 0.99	11
1.00 - 1.09	7
1.10 - 1.19	6
1.20 - 1.29	5
1.30 - 1.39	9
1.40 - 1.49	8
1.50 - 1.59	2
1.60 - 1.69	3
1.70 - 1.79	0
1.80 - 1.89	1
1.90 - 1.99	1

TABLE II. GROWTH OF HTNV AND APPEARANCE OF IF ANTIGEN IN A-549 AND E-6 CELL CULTURES

Time	PFU x 10^5		IF Antigen[a]	
h	A-549	E-6	A-549	E6
12	3.2	0.01	2+	-
24	10.4	0.01	3+	±
36	10.5	0.9	4+	2+
48	28.6	15.2	4+	4+
60	18.9	31.6	4+	4+
72	18.2	7.1	4+	3+

[a]-, no fluorescent cells; ±, less than 1 fluorescent cell per microscope field; 1+, 30 percent or less per field are fluorescent; 2+, 30-60 percent; 3+, 60-90 percent; 4+, greater than 90 percent.

TABLE III. SUMMARY OF OLIGONUCLEOTIDE HOMOLOGIES BETWEEN DEN-1 VIRUS STRAINS

Geographic Group	Strains (Year)		% Homology
Caribbean	Jamaica	('77) + *Bahamas ('77)	91
	Jamaica	('77) + Jamaica ('81)	86
African	Nigeria	('68) + Nigeria ('78)	79
Pacific/S.E. Asian	Bangkok	('74) + Bangkok ('75)	100
	Nauru No. 1	('74) + Nauru No. 2 ('74)	91
	Nauru No. 2	('74) + Hawaii ('45)	47
	Hawaii	('45) + Bangkok ('74)	49
	Bangkok	('74) + Nauru No. 2 ('74)	44
	Sri Lanka	('69) + Hawaii ('45)	38
	Sri Lanka	('69) + Nauru No. 2 ('74)	27
	Sri Lanka	('69) + Bangkok ('74)	21
Intrageographic comparisons	Hawaii	('45) + Jamaica ('77)	20
	Hawaii	('45) + Nigeria ('78)	20
	Bangkok	('74) + Jamaica ('77)	20
	Bangkok	('74) + Nigeria ('78)	24
	Nauru No. 2	('74) + Jamaica ('77)	30
	Nauru No. 2	('74) + Nigeria ('78)	30
	Sri Lanka	('69) + Nigeria ('78)	66
	Sri Lanka	('69) + Nigeria ('68)	60
	Sri Lanka	('69) + Jamaica ('77)	59
	Sri Lanka	('69) + Jamaica ('81)	55
	Jamaica	('77) + Nigeria ('78)	53
	Jamaica	('77) + Nigeria ('68)	49

*Plaque-purified large plaque virus.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DAOG 1529	82 09 30	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
81 10 01	D. CHANGE	U	U		NL	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AQ	197
b. CONTRIBUTING				
c. CONTRIBUTING	STOC 80-7.2:2			

11. TITLE *(Precede with Security Classification Code)*
(U) Enhancement of Host Defense Against Agents of Potential BW Importance

12. SUBJECT AREAS
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	2.2	1.445
c. TYPE	d. AMOUNT	83	2.6	1.596
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Physical Sciences Division, USAMRIID
b. ADDRESS *(include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR Wannemacher, Jr., R W
d. TELEPHONE NUMBER *(include area code)* 301-663-2833	d. TELEPHONE NUMBER *(include area code)* 301-663-7181
21. GENERAL USE FIC MILITARY/CIVILIAN APPLICATION:	f. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Neufeld, H A g. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Bunner D L

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Small Non-Protein Toxins; (U) Metabolism; (U) Rapid Detection; (U) T-2 Toxins; (U) Immunity

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Develop the capability of detection of small nonprotein toxins in environmental and physiological samples and evaluate prophylactic measures and/or rational means of treatment for U. S. Military forces who may be exposed to nonprotein toxins or infectious organisms.

24. (U) Develop animal models to study pathologic, biochemical, immunologic and physiologic alterations produced by toxins or infectious organisms. Use of toxin-haptene complexes to develop antibodies for rapid detection and evaluate the use of antitoxins or immunization to protect against intoxication. Develop procedure for extraction of toxins from environmental or physiological samples and detect them by biological, chemical, or immunological assays.

25. (U) 8110 - 8209 - A number of the high hazard diseases of BW potential do not have agent-specific vaccines or therapeutic treatment. To provide medical defense against these infectious diseases, studies have been involved in elucidating the pathophysiology and use of general supporting therapy in the treatment of these diseases. By use of nutrient and hormonal therapy, it has been possible to reverse the weight loss and protein wasting associated with infectious diseases. In addition, the therapy has been optimized to stimulate host defense mechanism and reduce the hepatic complication associated with many of these infections. An animal model has been developed to study hemorrhagic alteration that are associated with many of these infections and to develop possible therapeutic approaches. More recently studies have been initiated on the detection, pathophysiology, prophylaxis, and treatment of intoxications with the unique tricothecene toxins of BW importance. Publications: Biochem J. 190-663, 1980; JPEN 4:277, 1980; AM. J. Clin. Nutr. 34:229, 238, 1981; Antimicrob. Agents Chemother. 19:1042, 1981; Fed. Proc. 40:338, 525, 499, 862, 901, 919, 1981; Proc. Soc. Exp. Biol. Med. 166:6, 1981; Endocrinology 108(Suppl.):328, 1981; two book chapters.

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)

Work Unit No. S10 AQ 197: Enhancement of Host Defense Against Agents of Potential BW Importance

Background:

Threat analysis studies by the U.S. Army Medical Intelligence Agency have shown that certain of the small molecular weight, non-protein toxins are potential biological warfare agents which require medical defenses to protect our troops. One group of small non-protein toxins, trichothecenes mycotoxins, have been detected in environmental and biological samples in areas of Southeast Asia where tribesmen have been exposed to "yellow rain" (1, 2). To help in the development of medical defense against these toxins, it was necessary to develop a program for safe handling and decontamination; rapid detection and identification; determine the molecular mechanism of action; elucidate pathogenesis and physiological aberrations; develop methods of prevention, diagnosis and therapy; and evaluate potential aerosol threat. Initial studies have been limited to evaluation of trichothecene mycotoxin and marine toxins, saxitoxin and tetrodotoxin.

A number of high hazard diseases of BW potential dc not have agent-specific vaccines or specific treatments. To provide some medical defense, USAMRIID has been involved in elucidating pathophysiology and use of general supporting therapy in model systems which can be of value against highly hazardous diseases. A small effort has continued in this area under this work unit.

Progress:

1. Trichothecene Safety (Dr. R. W. Wannemacher, Jr.). Procedures for safe handling, decontamination, and stability testing of trichothecene mycotoxins have been studied. The FY 81 annual report (3) indicated that a SOP requires the use of surgical gloves, disposable gown, and eye goggles when handling liquid samples of small non-protein toxins and a full face respirator and a hood for powder samples of toxin. These regulations have been followed and no laboratory accidents have been observed in the past year. In addition, a semi-annual medical follow-up of all laboratory personnel is done, which includes complete hematology and clinical chemistry evaluation. Since some of these toxins may be teratogenic, pregnant females are not allowed to work with them.

2. Trichothecene Decontamination (Dr. R. W. Wannemacher, Jr.). To decontaminate this toxin, the FY 81 annual report (3) indicated that treatment of trichothecene (T-2 mycotoxins) with 2.5% sodium hypochlorite solution for 30 minutes was effective. When the cytotoxicity assay and TLC assay became available, additional studies were done to evaluate inactivation of T-2 toxin with sodium hypochlorite. In the process, a different batch of sodium hypochlorite was utilized in the studies, in which a 2.5% solution would not completely inactivate T-2 toxin in 16 hours, as measured by mouse lethal assay, rat and guinea pig skin test, cytotoxicity, or TLC assay. Differences between 2 lots of sodium hypochlorite could not be explained by available chlorine, which led to another study to evaluate sodium hypochlorite solution made alkaline with sodium hydroxide. A 2.5% sodium

hypochlorite + 0.25 N hydroxide solution completely inactivated T-2 toxin in 10 minutes by the mouse lethal assay, rat or guinea pig skin assay, cytotoxicity assay or by TLC analysis. A 1:10 dilution of this alkaline-hypochlorite solution resulted in a complete inactivation of T-2 toxin in 4 hours as measured by the above assay procedures. Based on these observation, a new SOP (15 Jan 1982) recommended that all spills and nonburnable waste including glassware, syringes, needles, vials, etc. be treated with 2.5% sodium, hypochlorite and 0.25 N sodium hydroxide by standing in the solution for 4 to 16 hours. All animal cages will be washed down with 0.25% sodium hypochlorite plus 0.025 N sodium hyroxide and allowed to soak for at least 4 hours before rinsing with water. In addition, the alkaline-sodium hypochlorite solutions are effective in neutralizing other trichothecenes including diacetoxyscirpenol (DAS) and deoxynivalenol (vomitotoxin).

In order to find a less caustic procedure for inactivation of T-2 toxin for use in aerosol chambers, formaldehyde, hydrogen peroxide, hydrogen peroxide exposed to UV light, and freon-113 plus acetic acid were evaluated as possible decontamination agents. None of these reagents inactivated T-2 toxin as determined by TLC, rat and guinea pig skin test, or cytotoxicity assay. A 0.2% acid (pH 5.2) sodium hypochlorite solution was slightly better as the neutralizer of T-2 toxin than hypochlorite alone but was not as effecitve as alkaline-hypochlorite mixture. Topical bleach, a mixture of calcium hypochlorite and alkali, was 100% effective in neutralizing T-2 toxin in 30 minutes, as was the currently utilized nerve gas decontaminant, DS-2. To date, the most effective neutralizing agent for decontamination of mycotoxins is alkaline-hypochlorite solutions. Because of the rubber seals and plastic windows, this agent could not be used in the aerosol P-3 cabinetry in Aerobiology Division. It was found that 2.5% sodium hypochlorite solution made from a stock 10 or 13% sodium hypochlorite solution that has been shown to be an effective neutralizer of T-2 toxin was a satisfactory decontamination agent when left in contact for 30 minutes in an aerosol cabinetry.

In general, trichothecenes are stable compounds which can be kept at room temperature in the dry state for a long time. T-2 toxin is stable in many of the lipid solvents at refrigerator temperatures for a long time. Preliminary, data suggest that this toxin may slowly break down in water solvents. T-2 toxin is not inactivated by autoclaving but is completely neutralized in temperatures in excess of 500 degree F. Thus, incineration (2200 degrees F) of burnable waste from mycotoxin studies is not only a feasible but recommended precedure in the SOP OF 15 January 1982. T-2 toxin is not neutralized by UV light or extreme high and low pH at room temperature. Solubility of T-2 toxin in water is approximately 0.5 mg/ml and an excess of 25 mg/ml in propylene glycol, and 250 mg/ml in methanol.

3. Trichothecene Detection:

a) Skin test assays: (Dr. R. W. Wannemacher, Jr.). The backs of rats, guinea pigs or rabbits are shaved and varied concentrations of the trichothecene in 2 microliter samples are applied to the exposed skin. Skins are evaluated on day 1, 2, and 3 after exposure and scored by technique of Draiz et al. (4). In rat and guinea pig, 10 ng of T-2 toxin can be detected with a 50% response dose (RD_{50}) of 60 ng. In contrast, the rabbit could detect 5 ng of T-2 toxin with an RD_{50} of 15 ng, which confirms earlier observation of increased sensitivity of the skin of the rabbit to the trichothecenes (5). In rabbits, detection limits for DAS was also 5 ng but was reduced to 20 ng for HT-2, 50 ng for T-2 triol, and 150 ng for T-2 tetraol. Thus, as the R groups of the trichothecenes became more saturated with OH

groups the sensitivity for a dermal response was markedly reduced. In contrast, the RD_{50} for the macrocyclic (Verrucarin A and rorodin A) was less than 2 ng. The skin test assay has proven to be very useful for measuring degrees of neutralization of the toxin by various decontamination agents. Because of the lack of specificity, they are of a lesser value for detection and identification of toxins in environmental or biological samples.

b) Cytotoxicity assay: (W. L. Thompson). Inhibition of protein synthesis in tissue culture cells by T-2 toxin using labeled amino acids has proven to be a simple, rapid, reproducible and sensitive technique for toxin studies. With this technique, it is possible to detect 3.5 ng of toxin per well or 70 ng per ml of starting solution. While this procedure does not have high specificity for a given trichothecene, it has proven to be an effective technique for screening decontamination agents, studies on neutralization of toxin acticity, and mechanisms of action.

c) Thin layer chromatography (TLC): (DR. J.G. Pace and C. F. Matson). Five (5) microliters of toxin solution is applied to a silica gel 60 TLC plate which is developed first with a solvent system of chloroform: ethyl acetate: ethanol (50:25:25) and a second time in the same direction with chloroform: ethyl acetate: ethanol (80:10:10). The developed TLC plate is stained with a 2% 4-P-nitrobenzyle (pyridine) and read on a gel scanner of a Beckman DU-8 spectrometer, utilizing white light. This procedure will effectively separate all of the trichothecenes that are presently available and has an effective detection of 0.1 micrograms per spot or 20 micrograms/ml of starting solution. This technique has been utilized effectively for measuring neutralization of the toxin with decontamination agents, distribution studies, and mechanisms of action.

d) Mycotox system: (MAJ J. O'Brien). The Mycotox System is a commercial instrument (Beckman) which utilizes inhibition of bacterial luminescence to detect toxic compounds. This system will detect 1 mg/ml of T-2 toxin and 1-10 μg/ml of deoxynevelenol (vomitoxin). It is currently being utilized to evaluate the toxicity of crude extracts of Fusarium fungi.

Gas liquid chromatography (GLC): (E. C. Hauer). Chromatographic profiles on 14 trichothecene derivatives have been obtained using Varian 760, glass column, with FID. A new gas chromatograph and associated data system have been ordered which is designed for a high resolution fused silica capillary columns and electron capture detection (ECD). The FID detector has a sensitivity of approximately 50 ng/ μl sample. This instrumentation is being utilized to analyze for parent toxins and their metabolic products in physiological fluids and tissues of experimental animals intoxicated with various trichothecenes. Techniques have also been developed for extraction and cleanup of samples for subsequent derivatization and analysis by GLC.

e) GLC/Mass spectrometry: (E. C. Hauer; Dr. E. P. Burrows, USAMBRDL; Dr. C. J. Mirocha, University of Minnesota). GLC/MS profiles have been obtained under a contract with USAMBRDL on 9 TFA and 6 TMS derivatives of trichothecene reference samples by electron ionization mode. Profiles were transferred to the search library. Seven TFA derivatives of the trichothecene reference samples have been

analyzed by chemical ionization (CI) mode in the range of 200 to 800 AMU. The CI specter have not been placed in the library as yet because of pressure-dependent relative ion intensity variations. When the CI spectral analysis is optimized, these and other spectra will be added to the library for automated identification of unknowns. Also, under this contract, analyses were made of 26 research samples expected to contain T-2, an isomer, or HT-2. The majority of these were extracts of the TLC plate scrapings. Five samples of T-2 incubated with glutathione were analyzed by direct inlet probe. Access via APL to FDA mycotoxin mass spectra file is now available through the USAMBRDL/FDA interagency agreement using the teletronics terminal in Bldg. 524. Under a contract with Dr. Mirocha with the University of Minnesota a mass spectral library of trichothecenes from analysis in his laboratory and that of FDA is currently being developed for use on the Hewlet-Packard 7906. This instrumentation is identical to the one utilized by Dr. Burrows at USAMBRDL and a disc containing 75 spectra in the library will be transmitted to USAMRIID to be added to the mass spec library. In addition, software have been written to allow transmission of data from terminals on the instrument at USAMBRAL to that of Dr. Mirocha's. Thus, cross-analysis of the sample can be done-via telephone communications between the 2 laboratories. Dr. Mirocha has a continuing effort to increase the trichothecene library by obtaining standards of Fusarium toxins from authors throughout the world and to establish collaboration with Israeli scientists in an effort to obtain toxins from Fusarium isolates originating in East Europe.

f) Immunoassays: (MAJ P. A. Fontelo; Dr. F. S. Chu, University of Wisconsin; Dr K. Hunter, USUHS). Dr. Chu has synthesized T-2 hemisuccinate-BSA haptenes which will stimulate specific antibodies in rabbits. Antisera produced in rabbits has been utilized to develop radioimmunoassays for the detection and quantitation of T-2 toxin. The assay is a competitive inhibition assay using ^{3}H-labeled T-2 toxin. Initially, the optimum antibody titer was determined by serial dilutions and adding trace amounts of radiolabeled T-2. Antibody dilution that result in a 50% binding of tracer was taken as an optimal titer and was used in subsequent assays. This antibody titer determined to be a 1:10 dilution. Although extremely low in titer, this is not inconsistent, since assays can be developed with low titer antibodies so long as their affinity constants are adequate. For this antibody, the affinity constant was shown to be 1.75×10^{10} L/mole. The range of detection is between 1 and 25 ng/ml. Cross reaction studies using other trichothecenes mycotoxins showed significant cross reaction with HT-2, 10.22%, while DAS and T-2 triol exhibited a cross-reaction of 0.1%. Other trichothecene tested, T-2 tetraol, Verucarol, vomitotoxin, Verrucarin A and roridin A did not show significant cross-reactivity. The interassay variation of coefficient is 9%. Biological samples such as whole blood, serum, urine and other specimens from decontamination protocols generated by other research units in this division have been analysed. Decisions and data interpretation have been based on the results of the assay. Blood and urine samples from Southeast Asia have been processed and tested using RIA procedure. The test has been in routine use for several months.

Using the same antibody an enzyme immunoassay (ELISA), has been developed to detect T-2 toxin. The antibody in optimal dilution is bound to 26 wells polyvinyl microtiter plates overnight. Peroxidase conjugated to T-2 hemisuccinate is made for the antibody competing sites with standard-T-2 or unknown serum or urine. The amount of bound T-2 peroxidase is reversely proportional to the concentratin of standards or unknown. Serum and urine samples with T-2 toxin added have been examined. Standard inhibition curve is somewhat flat with a rather high

background color that cannot be reduced further. This makes exact quantitation to unknowns difficult. Although ELISA is about 8-10 times more sensitive than the RIA, poor reproducibility and high coefficient of variation makes the test unsuitable for exact quantitation; however, it can still be used as a qualitative or semi-quantitative test for final quantitation done by RIA.

Dr. Chu is continuing to develop haptenes against other trichothecenes including vomitotoxin and DAS. These reagents are currently being evaluated for immunogenicity in the rabbits. In addition, he is attempting to make different molar ratior of toxin to carrier protein, as well as varying procedures for coupling the toxin derivatives to carrier protein. Besides bovine serum albumin, he is also evaluating the use of polylycine and thyroglobulin as possible carrier proteins. It is hoped that by varying the molar ratios, types of linkage protein, and protein carrier and he can come up with a haptene that is more antigenic than the one currently being utilized. Dr. Chu is also working on a new indirect ELISA for monitoring antibody titers against mycotoxin and for mycotoxin analysis. Specifically, he coats the plates with a mycotoxin-polylysine conjugate to which the T-2 specific antibody binds. This is then reacted with a second antibody, to which an enzyme is conjugated. The amount of goat anti-rabbit Ig-peroxidase bound to the rabbit Ig was subsequently determined by reaction with a chromogenic substrate. Free T-2 will displace the antibody from binding to the solid phase thus allowing for an assay for T-2 concentration. Although the indirect ELISA is very sensitive for monitoring a small amount of antibody present and also requires less antibody for analysis of mycotoxins, there are still some problems associated with this technique.

Because only low titer antibodies have been produced with extensive immunization in rabbits, attempts were made on the contract with Dr. Hunter to produce a monoclonal antibody. To date he has made 7 fusions following immunization of mice with the T-2 haptene to BSA, while some clones have produced an antibody that will bind to hemocyanine-T-2 hemisuccinate, the antibody is not displaced with free T-2. Dr. Hunter believes that part of the problem is related to the fact that T-2 toxin is immunosuppressive. He will be evaluating other haptenes made with less toxic trichothecenes.

4. Trichothecene Toxicity and Pathophysiology: (Dr. H. A. Neufeld, Dr. R. W. Wannemacher, Jr., LTC D. L. Bunner, CPT G. Parker, CPT L. Brennecke, LTC M. Cosgriff).

a) Mouse model: The LD_{50} for a subcutaneous injection of T-2 toxin in mice was 2.01 mg/kg body weight with 95% confidence limit of 1.63- 2.3 mg/kg. The mice died between 12 and 24 hours and necropsies indicated moderate to severe necrosis of lymphoid and epithelial tissue of small and large intestines and bone marrow, as well as lymphoid cells from lymph nodes, thymus, lung and liver. This mouse model has been utilized mainly to study neutralization of T-2 toxin by various agents and for screening of possible therapeutic agents for treatment of trichothecene intoxication. The LD_{50} for vomitotoxin by IP route was 43.5 mg, with a 95% confidence limit of 24.9-63.3 mg and by subcutaneous route was 44.7 mg/kg, with 95% confidence limit of 29.8-58.9 mg/kg. The majority of the mice died between 8 and 24 hours and necropsy data suggest mild to severe necrosis of spleen, lymph nodes and

gastrointestinal tract. Diffuse necrosis was noted in the bone marrow and mild multifocal congestion was noted in liver and kidney. While the histopathological lesions produced by T-2 and vomitotoxin was similar, the lethal toxicity of vomitotoxin was only 1/20th of that of T-2 toxin. In pigs, vomitotoxin was reported to have an emetic activity of 0.05 mg/kg. Since rodents do not exhibit emetic activity, it will be necessary to study this toxin in a species which is capable of emesis .

b) Rat model: Subcutaneously, intramscularly, or applied intranasally, the LD_{50} for T-2 was approximately 0.5 mg/kg. The LD_{50} for T-2 was 1.0, 1.8, and 2.2 respectively when given via intravenous, oral, or intraperitoneal routes. With the exception of the oral route, the rats died in 10-24 hours after exposure. When given orally, the rats died up to 72 hours postexposure. When applied to the skin in methanol, the LD_{50} was 8.65 mg/kg with deaths up to 7 days postapplication. When T-2 toxin was dissolved in DMSO, the toxicity was approximately 1.5 mg/kg in 24 hours. Thus, the route of administration as well as the vehicle utilized for applying toxin to the skin can influence toxicity. With the exception of the application of toxin to the skin in methanol, the histopathology was similar to the other routes of administration, characterized by a necrosis and depletion of lymphoid tissues of small and large intestines, lung, spleen, lymph nodes, bone marrow. In addition, there was severe necrosis and depletion of the mucosa of the small and large intestine. The rats developed severe hypothermia with a nadir at 6 hours; in surviving rats, body temperature became normal by 24 hours. Initially, the rats tend to hump up and remain that way for a long period of time with little movement. In most survivors there was diarrhea and severe anorexia for 3-4 days postexposure.

Regardless of the route of administration, T-2 toxin had no discernible effect on hemoglobin, red cell count, hematocrit or mean cell volume. Lymphocytosis was observed by 3 hours postinoculation. Rises in neutrophil population were also observed by 3 hours postinoculation, and counts remained elevated to 24 hours. Blood lymphocytes counts were initially elevated and then decreased to below control values at 18 and 24 hours postexposure. Platelet counts were depressed between 18 and 48 hours postinoculation.

One microgram of T-2 was required to produce a mild corneal injury and diffuse staining with fluorescein dye in the eye of the rat. Maximum effect was observed 24 hours after application of toxin and was resolved by day 3.

c) Guinea pig model: T-2 mycotoxin resulted in a LD_{50} of 1.04 mg/kg body weight, with 95% confidence limits of 083-1.19 mg/kg. The difference between the minimum effective dose and the LD_{100} was small. Clinical signs of intoxication include hypothermia, ascending paralysis, anorexia and weight loss. By 1 hour postinoculation of T-2 toxin, mild lymphoid necrosis was observed in the small and large intestine. Histopathological lesions became more severe with time, reaching a maximum at approximately 12 hours, which included severe necrosis and depletion of lymphoid tissue of spleen, lungs, lymph nodes, and intestine; necrosis of epithelial tissue of small and large intestine; depletion of bone marrow; mild necrosis of the liver, heart, kidney and adrenal cortex.

Hematological changes are characterized by increase in RBC, HGB, and HTC during the first 24 hours postexposure; appearance of reticulocytes during the first 12 hours; elevation in WBC, PMN, and lymphocyte for the first 12 hours followed by decrease in lymphocytes through 72 hours; and a slight decrease in platelets at 6 hours.

Initial studies on the effect of T-2 toxin in Hartley strain guinea pigs established that the toxin produced a marked coagulopathy. Within hours of toxin administration, the level of clotting factors begins to decline with the lowest levels reached on day 2 with return toward normal values on day 3. While the toxin does not produce thrombocytopenia, it does lead to markedly impaired platelet function.

Biochemical alterations include: elevation in serum glucose, blood urea nitrogen, uric acid, creatinine, triglycerides, potassium, phosphorus, copper, SGPT, SGOT, LDH, alkaline phosphatase, 5′ nuclosidase, CPK, hemolase, and free amino acids; and decrease in total protein, albumin, calcium, magnesium, zinc and iron.

From these observations, it is apparent that T-2 toxin causes marked destruction and depletion of rapidly dividing cells of the host, coagulation abnormalities, alterations in many metabolic pathways, and decreases in protein synthesis in cellular proliferation. All these effects could lead to a shock-like syndrome, which could be centrally mediated and may be the eventual cause of death. If guinea pigs survive for 24 hours, they will almost completely recover from the intoxication in 7 days.

d) Rabbit model: The rabbit has been utilized to measure T-2 toxicity when applied to the eye. Two micrograms of T-2 toxin were required to produce mild corneal injury in the rabbit. When larger doses up to 200 micrograms per eye were utilized, severe corneal damage was observed which was characterized by depletion of some of the cell layers of the cornea and underlying fibrous tissue. Additional studies will be required in this species to determine full effects of T-2 toxin on cornea and whether or not this process is a reversible phenomenon.

5. Trichothecene Distribution and Mechanism of Action:

a) Distribution studies: (Dr. J. G. Pace, Dr. R. W. Wannemacher, Jr.; C. J. Mirocha, University of Minnesota). In order to study the rate of metabolism and tissue distribution of T-2 toxin, guinea pigs in held metabolic cages were injected IM with an LD_{50} dose of labeled toxin. Blood was collected at 0., 3, 6, 12, 24, 48, 72, 168, 336, 672 hours after injection. Plasma was analyzed for radioactivity and concentration curve was used to determine biological "half life". The half time absorption of T-2 toxin to the blood is approximately 6 minutes. However, the half time of elimination from the blood appears to be biphasic. A first (early) half time was 1.8 hours and a second extended elimination phase had a half life of 49.5 hours. This may account for Mirocha's ability to detect trace levels of T-2 toxin metabolities in the blood of "yellow rain" victims 18 days after the actual attack (2).

After IM injection, T-2 toxin is rapidly eliminated into the feces and urine with a ratio of 1.16 at 24 hours. The actual amount of T-2 vs. other metabolites appearing in the elimination process has not been determined. By day 3, 50% of the total injected radioactivity had been lost via the urine and feces. Tissue distribution studies are still in progress. It has been determined that T-2

distribution in liver, lung, spleen, and adrenal was 3.5%, 0.35%, .07%, .02% of total radioactivity, respectively, 30 minutes after administration. By 30 minutes 3% of total radioactivity appears in the bile. T-2 triol was one of the metabolites identified by TLC in the bile sample.

Preliminary studies have been performed to determine the subcellular distribution of T-2 toxin in liver. Isolated rat liver was perfused in vitro with ^{3}H T-2 toxin for 5, 15, 30, 60, and 120 minutes. The livers were homogenized and fractionated on a discontinuous Percoll density gradient (6). After 2 hours of perfusion with T-2 toxin, 43% of label was associated with the endoplasmic reticulum, and 4, 8, and 4% as associated with the plasma membrane, lysosome, and mitochondria rich fractions, respectively. Only 1-2% was found in the nuclear fraction and the remaining 40% was cytosolic.

At the University of Minnesota, an effort has been initiated to obtain sufficient metabolic products of T-2 toxin, and the TC-6, TC-3, and TC-1. After deliberation on methodology, it was decided to use the cow because of the volume of urine and feces generated per unit time. It is hoped sufficient quantities of the above metabolities will be obtained in order to identify the products. Once these metabolities have been identified and characterized, they will be incorporated into the mass/spectrometry library.

The cow will also serve as a model to study kinetics of T-2 toxin metabolism. An important facet of this study is the kinetic response of T-2 metabolism as monitored in the blood of a cow. It is planned to study the residue of T-2, namely HT-2 and T-2 tetraol in order to determine the half life of T-2 toxin when ingested. The primary objective is to determine whether exposure of individuals to T-2 toxin can be detected by analyzing blood for trace residues. Trace amounts of T-2 toxin and its metabolities have been detected in whole blood of victims exposed to "yellow rain" in Southeast Asia (2). These kinetic studies will be pertinent in the above findings and the controversy that exists in determination of the half-life of T-2 mycotoxin. Principles uncovered in this study will aid in designing similar efforts involving the metabolism of diacetoxyscirpenol.

b) Mechanism of action: (Dr. J. G. Pace, W. L. Thompson, Dr. J. Middlebrook, MAJ J. O'Brien, Dr. L. C. Sellin). In vitro and in vivo studies have shown that T-2 toxin impairs energy production at the cellular level. Site of inhibition has been identified as site I of the electron transport chain. Many toxins are detoxified by reactions involving glutathione. It is important to study plasma, biliary, and tissue levels of glutathione conjugates. Spectrophotometric assay has been developed at USAMRIID and plasma and bile levels are being detected in rats exposed to T-2 toxin. In the isolated perfused liver system, biliary glutathione remains constant over 1 hours perfusion time when perfused (recirculating system) with an LD_{50} dose of labeled T-2 toxin. Fifty percent of the label is taken up by the liver in 30 minutes. In the same time frame, 50% of the label that was taken up by the liver is excreted into the bile. Bile samples were analyzed by TLC and bioscan radioisotopic scaning. The metabolities were identified as HT-2, and T-2 tetraol and 3-4 more polar unknown metabolites. In a non-recirculating perfusion system the uptake of T-2 toxin is linear with time at 50% of the toxin is removed on each passage from 5-60 minutes. In the perfusion system T-2 toxin does not appear to be bound to plasma albumin but remains with the perfusate buffer on passage through a G-25 column.

The effect of T-2 mycotoxin on the viability of Vero cells was studied using trypan blue excusion and plating efficiency studies. With up to 3 logs over the LD_{50} for protein synthesis inhibition and 1 hour exposure to the toxin, the cells showed no difference from controls in either trypan blue exclusion or plating efficiency. Although the protein synthesis was supressed at the time of removal of the toxin, recovery of the protein synthetic ability of the cells took place with time. Also, protein synthesis in repeated cells 24 hours after toxin exposure was suppressed only in cells exposed to very high doses of T-2 toxin (25 µg/ml). All this information indicates that at cellular level T-2 toxin caused a temporary shutdown of some metabolic functions but not permanent damage.

Nine different trichothecenes having the same basic structure, but differing in slide group attachment at 5 different locations on the ring structure were tested for their relative levels of protein synthesis inhibition in Vero cells. Based on these results from structure of various derivatives, the existence of a side group on carbon 15 is of major importance in the toxicity of the molecule while position 3 is also, but to a lesser degree, important. If, as it is thought, all these derivatives bind equally well, the existence of the side chains at strategic locations is probably necessary to interfere with some part of the translational process.

Using ^{3}H-leucine as marker, cell culture studies have shown that there is an overall suppression of amino acid uptake 10 to 15 minutes after addition of a high dose of T-2 toxin. At the same time, amino acid incorporation to TCA percipitable fraction is completely shut down allowing for a buildup, over control levels, of labeled leucine in the TCA soluble fraction. A similar pattern is noted with DAN replication precursors using H3 thyidine, except that a buildup in the soluble fraction occurs in response to T-2 toxin.

Labeled T-2 toxin is taken up rapidly by Vero cells, the rate and quantity bound at equilibrium is directly proportional to the concentration of toxin added. Although the TCA precipitable fraction is also dependent on concentration for the amount bound per unit of time, the amount of toxin bound, particularly in lower concentrations of labeled T-2, begins to level off at 30-40 minutes after exposure. Only about 1% of the total radioactivity is found in the TCA precipitable fraction.

Release of labeled T-2 toxin from the total TCA soluble and TCA precipitable fractions of Vero cells occurs rapidly for the first several hours and then begins to slow down, probably due to free diffusion in and out of the cells of previously released toxin. However, the addition of cold T-2 to the media causes an even more rapid and extensive release of labeled material in the total TCA soluble fractions than in its absence. No difference was noted in the TCA precipitable fraction. This would argue against free diffusion of the toxin, and more toward displacement of labeled toxin by cold toxin, since the labeled toxin will be working against the concentration gradient by moving more rapidly out of the cell. Further studies will be conducted to better interpret these results.

Utilizing labeled T-2 toxin in studies with the Chinese hamster ovary (CHO) cell line, it was determined that the kinetics of T-2 cell at both 4 and 37°C exhibited typical biomolecular reaction patterns increasing to and maintaining a steady-state plateau. At 4 degrees, equilibrium was reached in approximately 6-8 hours, whereas at 37 degrees, equilibrium occurred by 1-1 1/2 hours. Not only were

the kinetics of T-2 toxin cell association more rapid at higher temperatures, the equilibrium state was 4-5-fold higher at 37° than 4°. When unlabeled T-2 toxin was added to the label product at a 100-fold molar excess in the kinetic studies, toxin-cell association at both 4° and 37° was blocked by 95-98%. These observations would suggest that the plasma membranes act as a barrier to intracellular transport of T-2 toxin. If the toxin entered the target cells by simple diffusion, one should not obtain competition because the fusion is not a process. In addition, when the naturally occurring biological active form (α-T-2) was compared to the enantiomer and biologically inactive form (β-T-2 toxin), it was found the alpha isomer was taken up by CHO cells at a rate 50 times that of the beta. These results suggest a steriospecificity of the uptake process, a feature certainly not expressed by diffusion.

By "Scatchard plots" or 50% blocking of binding of radiolabeled T-2 toxin, it was determined that the association constant for cell binding was 2.6-3.0 x 10^8 M and a number of binding sites per cell at 3-6 x 10^6.

A T-2 toxin resistant isolate of a CHO cell line was produced by treatment with ethyl methane sulfonate followed by exposure to toxin for several weeks. This isolate was cloned by limiting dilution, which produced 14 T-2 resistant sublines, which were frozen in ampules to assure seed stocks. A 2.5-3-fold resistance was observed with all of the cell lines cloned and frozen as seed stock. While not a large factor of resistance, the effect was very easy to document and was more than adequate to allow further study.

It was noted that the dose-response curve for T-2 toxin inhibition of DNA synthesis was shifted by exactly the same factor as for that of protein synthesis. These observations would suggest that resistant cells with 2 mutations have been produced, one in the DNA and one in the protein biosynthetic mechanism, or that direct inhibition of protein synthesis led indirectly to inhibition of DNA synthesis. Since both cycloheximid and puromycine, well established to act on protein synthesis, both qualitatively and quantitatively produce similar effects on protein and DNA synthesis as seen with T-2 toxin, it was concluded that the toxin does not directly inhibit DNA synthesis.

In testing the pattern of cross-resistance of other trichothecenes mycotoxins, it was observed that the T-2 resistant mutants prove resistant to roridin A, verrucarin A, HT-2, T-2 triol, and deoxynivalenol but not to T-2 tetraol. When the various trichothecene toxins were utilized to block cell association of radiolabeled T-2 toxin, it was found that roridin A, verrucarin A, and T-2 were very effective competitors, T-2 triol and deoxynivalenol were less potent and T-2 tetraol had little or no effect. This pattern would strongly suggest a specific but common binding site for all of the trichothecenes tested, with the exception of T-2 tetraol.

T-2 mycotoxin will inhibit protein synthesis in isolated hepatocytes. The LD_{50} for protein inhibition is similar to that observed in other cell lines, such as the Vero cells. By 4 hours contact with the T-2 toxin, liver enzymes such as LDH, SGOT, and SGPT start to leak out into the medium. By 12 hours, there is a 40-fold increase in the concentration of these enzymes in the media, suggesting severe cellular damage.

In studies with isolated muscle fibers both *in vivo* and *in vitro*, treatment with T-2 toxin resulted in an increase in membrane potentials. This would suggest

that T-2 toxin is perhaps stimulating the sodium potassium pump. At this time it is not possible to relate these observations to the ascending paralysis observed in the guinea pig.

6. Trichothecene Prophylaxis and Therapy:

a) Cell culture: (W. L. Thompson, MAJ J. O'Brien, Dr. J. G. Pace). High doses of cystine, glutathione, and cystamine resulted in a 50% inactivation of T-2 toxin as measured by its effect of inhibition of protein synthesis in Vero cells. Ser m from normal and turpentine-inflamed rats, without modification or after heat inactivation (56 degrees for one hour), was tested and compared to normally-used heat-inactivated fetal calf serum. Very little difference in the 50% protein inhibition was noted in either pretreatment of the T-2 mycotoxin or exposure of tissue culture cells to the different control serums. However, small increases were seen in T-2 neutralization when a toxin was pretreated with inflamed serum. More pronounced results may be seen in a primary hepatic cell culture.

One of the trichothecene derivatives, verrucarol, has very little effect, if any, on protein synthesis inhibition in tissue culture cells. However, since verrucarol still has the same basic ring structure, including the epoxide group, as the other trichothecenes, studies were conducted to see if verrucarol could be used as a competitive inhibitor of T-2 toxin. Cells were incubated for 30 minutes with verrucarol and then challenged with T-2 mycotoxin. Relatively good protection from T-2 toxin (75-86% inhibition) was seen with doses of 10 g/ml of verrucarol. Subsequent experiments, with verrucarol removed from the cells prior to challenge with T-2, showed that any protective effect was removed with a 30 minute wash prior to T-2 challenge. Therefore, it appears that the protective effect is dependent on the presence of the inhibiting molecules. Feasibility of this approach using neutralizing mycotoxin as a form of prevention or therapy is being investigated in the mouse model.

Cultured hepatocytes have been utilized to study the effect of glutathione and cysteamine on T-2 toxicity. As with the Vero cells, both compounds decreased the effective dose 50 (ED_{50}) for T-2 toxin. This would suggest that cultured hepatocytes can be used as a preliminary screening system prior to screening drugs for therapy in animal models.

b) Mouse model: (LTC D. L. Bunner, CPT R. Fricke, Dr. J. G. Pace, Dr. R. W. Wannemacher, Jr.). Because of the number of animals required, the mouse model for T-2 intoxication was selected to screen compounds of potential value in prophylaxis or treatment studies. Two radioprotective compounds, glutathione and cysteamine, have been screened for their protective effect in mice exposed to T-2 toxin. These compounds appeared to increase, rather than decrease, the toxicity of T-2 toxin in mice. Both the LD_{50} and mean time to death were decreased. The results correlated with the protein synthesis inhibition studies in cultured hepatocyte system. Diehylmalate, a glutathione depleter, also increased the death rate due to T-2 toxin.

When verrucarol was given at 10 mg/kg i.p. 1 hour before a s.c. injection of 2.5 mg/kg of T-2 toxin, all of the mice were dead within 12 hours. The mice injected with T-2 toxin or verrucarol alone were alive at 24 hours. At necropsy, T-2 toxin treated mice showed marked histopathology and depletion of

the bone marrow. In contrast, no histopathology was associated with the mice given verrucarol alone. These preliminary results would suggest that pretreatment with a non-toxic trichothecene that can compete for the binding sites with T-2 toxin can increase the lethality of T-2 toxin when given one hour prior to challenge. Future studies will evaluate the use of verrucarol when given shorter time periods before challenge with T-2 toxin.

c) Rat skin model: (Dr. R. W. Wannemacher, Jr., LTC D. L. Bunner, R. E. Dinterman). Since skin absorption could be one of the major routes of administration of toxins in aerosol exposure, different cleansing agents such as water, soap and water, diatomaceous earth, chemical warfare decontamination kits, commercially available towellettes and washcloths were utilized to decontaminate the skin of rats at various times after exposure to T-2 toxin. With the exception of wiping with diatomaceous earth, all the treatments utilized in these studies were effective in decontaminated skin immediately after exposure. Most of the treatment procedures became less effective with time after exposure. However, even at 6 hours after exposure a number of procedures were able to remove 60-100% of the toxin from the skin. Treatment with soap and water, towelletes, or disposable washcloths were effective in removing T-2 toxin from the skin and did not produce noticeable toxic effects. At later time periods, treatment with water wash, water rinse or wiping with diatomaceous earth was not a highly effective treatment for decontamination of the skin, after T-2 toxin exposure. The M-258A-1 decontamination kit resulted in the highest percentage of removal of toxin from skin. The procedure was not significantly better than the use of M-258 decontamination kit, towellettes, disposable wash cloths or soap and water. Reagents in the decontamination kit did not inactivate T-2 toxin in an in vitro system, which would suggest that the mechanism of action is by a mechanical cleansing of the skin to remove the toxin. Also, these kits, especially the M258 kit, were toxic to the rats and did cause some skin irritation and lethality. This effect can be partially reduced by the use of more dilute kit material and rinsing with saline after application of the decontamination solutions.

In the above study, toxin was applied to the skin in either methanol or ethanol:glycerol:water (2:3:5) which are slowly absorbed in systemic circulation. If toxin is applied in DMSO, it is rapidly absorbed through the skin and lethality response is much greater than that with other lipid solvents. When a lethal dose of toxin in DMSO is applied to the skin, it could be removed after 5 minutes by washing with soap and water but not after 1 hour. These data suggest that immediate washing of the skin with soap and water markedly decontaminated the skin and reduced lethal effects of T-2
toxin. As such, it would appear to be an effective immediate treatment for topical exposure to "yellow rain", even if DMSO were used as a vehicle. Further studies will evaluate the use of soap and water to decontaminate the skin following exposure to crude toxic trichothecene extracts.

7. Trichothecene Aerosol exposure: (Dr. R. W. Wannemacher, Jr., CPT R. Fricke, LTC D. L. Bunner, COL E. Stephenson, R. E. Dinterman). In a preliminary study, rats were exposed to a T-2 aerosol using a modified Henderson aerosol apparatus contained in a class III biological safety cabinet system. The T-2 toxin was dissolved in methanol:glycerol:water (3:2:5) solution at a concentration of 6 mg/ml. Eight rats each were exposed for 10 or 20 minutes to the aerosol. This was followed by a 10 minute air wash after which the rats were removed from the exposure

chamber and observed for 7 days. The exposure rate as determined by a cotton sampler was 0.039 and 0.062 mg/kg body eight for the 10 and 20 minute study, respectively. Part of the difficulty with the study was that the T-2 tended to be more soluble in the glycerol, which was not readily aerosolized. As a consequence, the exposure dose was too low and none of the rats died or showed any signs of clinical illness.

In a second study, propylene glycol was chosen as a solvent for the T-2 toxin. Propylene glycol is readily aerosolized and does produce an aerosol with an average particle size of 3 microns. Eight rats were exposed for 20 minutes to an aerosol produced from a solution that contained 25 mg of T-2/ml of propylene glycol. The calculated inhalation dose was 0.68 mg/kg body weight. None of the rats died but they did show some clinical signs including anorexia, reduced fluid intake, and irritability. From these preliminary studies it may be concluded that aerosol exposure was not a markedly more lethal means of delivering T-2 toxin than systemic exposure.

8. Marine toxins (saxitoxin and tetrodotoxin)

a) Safe handling, decontamination, and stability:
(Dr. R. W. Wannemacher, Jr., R. E. Dinterman). Similar handling procedures to those described for mycotoxins have been employed for the last year with marine toxins. Both tetrodotoxin and saxitoxin are readily inactivated by sodium hypochlorite at the concentrations utilized in the mycotoxin studies. Therefore, the SOP of 15 January 1982 is effective for the safe handling and decontamination of both the mycotoxins and marine toxins.

In studying the kinetics of sodium hypochlorite inactivation tetrodotoxin, it was found that in 30 minutes, 75 parts per million of chlorine are required to inactivate 50% solution that contains 100 parts per million of tetrodotoxin, while it took 56 parts per million of chlorine to inactivate a similar amount of toxin in 4 hours. Ten parts per million of hypochlorite or tap water did not inactivate tetrodotoxin in 4 hours . Thus, ordinary chlorination in drinking water would probably not inactivate tetrodotoxin. Exposure of tetrodotoxin to pH's between 2.7 and 8.0 did not cause any inactivation at room temperature for 4 hours. Exposure to pH 2.2 or 9.2 resulted in 50% inactivation in 4 hours. Fifty percent of tetrodotoxin was inactivated when exposed to 250 degrees F for 30 minutes and complete inactivation was observed with autoclaving or with temperatures over 270 degrees F. An exposure to 12,000 parts per million of hydrogen peroxide for 16 hours inactivated 50% tetrodotoxin. In contrast, only 50 parts per million of hydrogen peroxide were required to inactivate 50% of the tetrodotoxin with simultaneous exposure to UV light for 16 hours. Exposure of tetrodotoxin to UV light for 16 hours in itself resulted in a 30% inactivation of the toxin. Thus, tetrodotoxin is relatively stable and is more rapidly inactivated by sodium hypochlorite, UV light, or heat than are mycotoxins.

Exposure of 50 parts per million of saxitoxin to 22 and 14 million parts per million hypochlorite for 30 minutes and 4 hours, respectively, resulted in a 50% inactivation of toxin. When exposed to 10 parts per million of hypchlorite or tap water for 4 hours, saxitoxin was not inactivated. This toxin was stable in pH range between 2.4 and 11.7 for 4 hours. At a pH of 1.5 or 12.2 50% of the toxin was inactivated in 4 hours. Saxitoxin was slowly inactivated at temperatures above 100 degrees F with 50% inactivation at 250 degrees F. Toxin was completely destroyed by

autoclaving or temperatures in excess of 270 degrees F. A 16 hour exposure to the toxin to 25 parts per million of hydrogen peroxide resulted in 50% inactivation of saxitoxin, while only 3 parts per million were required in the presence of UV light. UV light for 16 hours resulted in a 7% inactivatin of saxitoxin. The saxitoxin was more stable at higher pHs than tetrodotoxin but was slightly more sensitive to inactivation by hypochlorite or temperature. Hydrogen peroxide was much more effective in activating saxitoxin than tetrodotoxin but it was less sensitive to UV light.

b) Detection:

(1) Mouse bioassay: (Dr. R. W. Wannemacher, Jr., CPT S. R. Davio, R. E. Dinterman). The LD_{50} for intraperitoneal injection of tetrodotoxin in the mouse was 14.2 micrograms/kg with 95% confidence level of 12.1-18.9 micrograms/kg. When tetrodotoxin was injected subcutaneously the LD_{50} was 22.5 micrograms/kg, with 95% confidence levels of 18.8-24.9 micrograms/kg. For both routes of administration of a toxin, a linear correlation was observed between the mean time to death and the log dose. Regression analysis had a P value of less than 0.01 and an effective range from 15-500 micrograms/kg.

For saxitoxin the LD_{50} by the intraperitoneal route was 24.4 micrograms/kg, with 95% confidence level of 15.9-31.0 micrograms/kg. By subcutaneous route the LD_{50} was 15.7 micrograms/kg with an LD_{50} confidence limit of 13.1-20.9 micrograms/kg. A highly significant correlation was observed between the mean time to death and the log of dose of saxitoxin, through a dose range of 15-1000 micrograms/kg. For both tetrodotoxin and saxitoxin, the use of the mean time to death proved to be an effective procedure for quantitation of the amount of toxin in a given sample. If the mouse did not die in 15 minutes, the sample was considered to be nontoxic.

(2) Receptor binding assay: (CPT S. R. Davio). Isolated rat brain membranes are utilized to measure binding of ^{3}H- saxitoxin to a specific receptor in the sodium channels. Unlabeled saxitoxin and tetrodotoxin will displace the binding of the tritiated saxitoxin on the receptor membranes. This binding assay can detect 1 ng quantities of saxitoxin in buffer, urine, or plasma, and 10 ng of tetrodotoxin in buffer. Once prepared, the rat brain membranes are stable for at least 6 months when stored at -70 degrees. This is a highly sensitive assay which is capable of detecting small quantities of marine toxins in physiological fluids. This assay appears to be specific for saxitoxin and tetrodotoxin.

(3) Fluorescence assay: (CPT S. R. Davio). Treatment of saxitoxin with hydrogen peroxide converts it to a fluorescent compound which can be detected with a sensitivity of approximately 50 ng. It was not possible to detect the saxitoxin in unextracted samples of urine or plasma. While this procedure is relatively sensitive, it does not appear to be a usable technique for detection in physiological fluids unless the toxin is extensively extracted and purified.

c) Toxicity and pathophysiology: (Dr. H. A. Neufeld, Dr. R. W. Wannemacher, Jr., CPT L. Brennecker). In mice treated with tetrodotoxin, the only histopathology was hemorrhage and congestion of the lung and hemorrhage of the thymus. No discernible histopathology was observed in mice that died in less than 15 minutes from saxitoxin.

d) Prophylaxis and therapy: (CPT S. R. Davio, Dr. L. C. Sellin). A drug screening program has been initiated to identify drugs which could be potential antidotes against marine toxins. When fully operational, this program will utilize ^{3}H- saxitoxin receptor binding studies, rat muscle electrophysiologial studies, and whole mouse bioassay to evaluate drugs for their therapeutic efficacy. The tritiated saxitoxin-receptor binding assay and mouse bioassay have already been utilized to evaluate several drugs which in the old literature were suggested as being effective antidotes against saxitoxin and tetrodotoxin. It was concluded from these studies that pentylenetrazol, caffeine, diethylnicotinamide, or lobeline were totally ineffective as antagonists of the effects of saxitoxin or tetrodotoxin. Further, the antidotes used against chemical nerve gases, atropine and 2 PAM, did not afford any protection (alone or in combination) against toxic effects of saxitoxin.

9. Sandfly Fever Studies in Man: (LTC D. L. Bunner).

A total of 6 volunteer subjects was studied, 3 males and 3 females. Five subjects became ill and using analysis of variance of repeated measurements showed clear-cut changes in plasma growth hormone concentration levels although no alterations occurred in prolactin responsiveness. This is an important finding for several reasons. It does verify previous reports showing elevated growth hormone values during glucose tolerance tests in Sandfly fever. It also shows that during spontaneous and normal feeding during this infection, growth hormone values are clearly altered as well. In spite of the marked elevation in growth hormone in Sandfly fever, body nitrogen loss is a usual concomitant event. One must then postulate a resistance growth hormone caused perhaps by inhibitors of growth hormone, or perhaps by alterations in growth hormone binding or post binding events in target cells.

An artificial hyperthermia study was done to document hormonal responsiveness in the same subjects to elevated body temperature in the absence of infection. Qualitatively and quantitatively, it was clear that growth hormone responses were similar to those seen during Sandfly fever infection. Prolactin levels again did not change. One would have to postulate that the elevated body temperature per se at least contributed significantly to the growth hormone response seen during infection

10. Sandfly Fever Studies in Non-human Primates: (LTC D. L. Bunner).

Cynomolgus, rhesus, and African green monkeys were evaluated as possible non-human primate models for Sandfly fever. Following inoculation of the virus, the monkeys did not have any significant change in body temperature, food intake, or other clinical signs of illness. Blood samples are awaiting analysis for determination of seroconversion and viremia. If the monkeys do seroconvert or show evidence of viremia, additional studies will be considered.

11. Malaria in Man: (LTC T. M. Cosgriff).

As an additional study component of ongoing antimalarial drug tests in volunteers, platelet-associated IgG and complement were measured in 5 subjects with induced malaria. None of the volunteers have been previously exposed to malaria. While all of the subjects developed varying degrees of thrombocytopenia, neither immnoglobulins nor complement were detected in the subjects' platelets in concentrations above those found in controls. While platelet-associated IgG has been implicated in the pathogenesis of thrombocytopenia and other infectious disease, these findings argue against its importance in malaria.

12. Pichinde Virus in Guinea Pig: (LTC T. M. Cosgriff).

Initial studies of the Pichinde virus infection in strain 13 guinea pigs (a model system for studying hemorrhagic viral diseases) established that such infections produced significant hemostatic derangement including decreased concentrations of multiple coagulation factors, decreased platelet function manifested by impaired whole blood platelet aggregation, and thrombocytopenia. Infected guinea pigs also developed hemoconcentration manifested by increased hematocrit values, as well as neutrophilia and lymphopenia. Fibrinogen concentrations do not decrease appreciably in infected guinea pigs and fibrinogen degradation products cannot be detected in the serum, which is evidence against the occurrence of intravascular coagulation. Addditional studies are planned to further characterize the effects of Pichinde virus infection in this model and to elucidate the mechanism of such effects.

13. Antithrombin III from Cryoprecipitate Human Blood: (LTC T. M. Cosgriff,).

Because antithrombin III can be severely depleted in conditions such as disseminated intravascular coagulation, and because its replacement may be an important part of therapy, the antithrombin III content of cryoprecipitate was measured to see if it could serve as a source of antithrombin III. It was found that the concentration of antithrombin III in precipitates was no higher than that of normal plasma. Further, heparin had no effect on the antithrombin III content of the cryoprecipitates.

14. Model Streptococcus Pneumoniae Infections:

a) Nutrient support therapy in non-human primates: (Dr. R. W. Wannemacher, Jr., LTC D. L. Bunner). The tethered jacketed monkey model was utilized to evaluate the effect of nutrient support therapy and/or sepsis on plasma albumin protein dynamics. Intravenous infusion of amino acids and/or dextrose has marked effects on the rate of synthesis of mixed plasma and albumin proteins. While detrose infusion tended to conserve body protein by decreasing the rate of turnover of total body protein, it markedly reduced the fractional rate of synthesis of plasma albumin. An infusion of amino acids resulted in a greater turnover of body protein but also had a marked stimulatory effect on synthesis of albumin and albumin concentrations. Amino acids plus detrose infusion tend to intermediate between two other nutrient support therapies. Pneumococcal sepsis had no effect on the rate of synthesis of plasma albumin but markedly increased its rate of breakdown. A combination of reduced synthesis and increased catabolim resulted in a marked decrease in plasma albumin content in infected monkeys that were infused with dextrose. These observations raised the question as to whether measurements of plasma albumin can be used as a criteria for assessing the value of nutrient support therapy for stimulating host defense against infectious disease.

b) Hormonal response in the septic rat model: Insulin binding to different cells during sepsis in the rat:(MAJ G. A. Saviolakis and LTC D. L. Bunner). An insulin binding radioreceptor assay has been developed for the following cell or tissue receptor systems: cultured human lymphocytes, isolated rat hind limb muscles, rat spleen lymphocytes, and rat liver membranes. A circulating inhibitor of insulin hormone binding to receptors on cultured human lymphocytes was not found in the serum or plasma of Streptococcus pneumoniae-infected rats.

Pneumococcal infection was induced in 80-100 g rats by subcutaneous injection of 10^4 microorganism/kg and the soleus and extensor digitorum longus muscles were studied at 24 hours (early phase) and 48 hours (peak phase) after infection using the insulin binding assay. At 24 hours there was no difference in the insulin binding between infected and control fasted rats. At 48 hours, however, insulin binding to both the soleus and extensor digitorum longus muscles increased by 20-30%. This effect was predominantly due to increased numbers of binding sites. Serum plasma glucose values were modestly elevated and insulin concentration increased 2-fold by 48 hours, a combination suggesting presence of insulin resistance. The finding of increased insulin receptors suggest the presence of post-receptor defects in insulin action in muscle from infected rats.

In the same experimental model, insulin binding was increased to spleen lymphocytes isolated from infected rats. With these cells, enhanced binding was observed at 24 hours and 48 hours after infection and was due to increased receptor concentrations. In studies on isolated liver membranes, insulin binding was not affected by 24 hours in septic rats but was decreased at 48 hours due to a decrease in receptor number. Further studies are underway in these 3 receptor preparations to obtain enough data for regular statistical analysis and to correlate the receptor binding changes to post-receptor events, i.e. generation of hormonal intracellular messenger, nutrient transport, and enzyme activity. Data collected to date indicate that the membrane receptor for insulin undergo changes during infection which could be partly responsible for the observations of altered nutrient metabolism. In addition, enhanced binding of insulin to lymphocytes may be the result of their activation during infection.

c) Insulin therapy: (LTC D. L. Bunner).

Insulin is readily available and there is some prior evidence in burn patients suggesting that insulin could provide a genuine anabolic effect during illness. A pilot trial using rats was carried out which showed smaller weight loss in insulin treated infected rats. Normal rats given parenteral insulin gained 15.75 grams over a 3 day period. Pneumococous-infected rats given insulin however, lost 1.14 grams over the same time period. This small loss compared well with that of infected rats not given insulin which lost 14.75 grams during the same period. However, there was an excess mortality in the insulin treated group as their appetite had not improved. Clearly, parenteral supplementation will be required for this type of study. Mild hypoglycemia can serve as an appetite stimulus but apparently this did not occur in the infected rats.

d) Hormonal regulation of inhibition of ketosis in the septic rat: (Dr. H. A. Neufeld, LTC D. L. Bunner). In the fasted rat that has an infection or inflammatory stress imposed upon it, there is a negative correlation between the plasma concentrations of insulin and the plasma concentration of ketone bodies. When ACTH or corticosterone were administered at 1-2 mg/kg to 12 hour fasted rats, plasma ketones were depressed and insulin concentrations elevated. Corticosterone could produce this effect in adrenalectomized rat but the response was not observed when ACTH was administered to adrenalectomized rats.

If fatty acids were perfused through the isolated liver of fasted rats in the presence of physiological concentrations of glucose, there was no impedance of ketone body production. Similarly, if the fatty acids were perfused in the presence of high concentrations of insulin, the same lack of effect was seen. However, if the fatty acids were perfused in the presence of physiological concentrations of both glucose and insulin, there was a decided reduction in rate of ketone body production.

All of the work accomplished so far points to a complicated endocrinological response to infection and/or inflammation. The sequence of events seem to be as follows: Any infection or inflammatory stress results in a release of some signal which stimulates the hypophysis to release ACTH, the adrenal to release glucocorticoids, and the pancreas to release both glucagon and insulin. It is the insulin or some unknown secondary factor stimulated by insulin which also may be responsible for the inhibition of ketosis in the septic host.

15. Effects of Exercise on Infection: (N.-G. Ilback, University of Uppsala, LTC A. J. Johnson and Dr. W. R. Beisel).

The effects on various infections of prior physical conditioning and of acute exhaustive exercise were studied in animal models. These modes included S. typhimurium studies in rats and influenza and F. tularensis studies in mice. These research efforts extended earlier observations made during tularemia in rats, in which daily swimming exercise in infected rats caused relatively little exacerbation of illness severity. However, the tularemia infection reduced physical performance capacity of rats but not the normal "training" response to daily exercise.

When tularemia was studied in mice, daily exhaustive swimming in mice also counteracted many of the infection-induced proteolytic and other biochemical changes in the myocardium, but it did not alter the severity or progression of the infection. Physical preconditioning also limited the catabolic response without changing the outcome of the infection. In contrast, an influenzal infection was less severe in the exercise preconditioned mouse, but was of greater severity in mice forced to exercise during the viral illness. As an additional finding in the absence of exercise, myocardial muscle protein degradation appeared earlier and was more pronounced in mouse influenza than in mouse tularemia of equal lethality. A number of other differences in myocardial enzyme responses were also evident when the two infections were compared, although neither of these infections led to myocarditis.

A different model was also studied, i.e., rats infected with S. typhimurium with or without the added stress of exercise in running wheels. In adult rats the infection caused a metabolic impairment, i.e., in reduced protein synthesis and oxidative capacity, in both skeletal and myocardial muscle, although the decrements were greater in skeletal muscle. In young weanling rats, the same infection induced a transient myocarditis, with increases in myocardial protein, nucleic acids, and lipids, and in the activities of several enzymes. Acute exercise during the infection caused an additional increase in myocardial protein.

In combination, these studies appeared to indicate that prior physical conditioning exercise improved host resistance to infections. However, infections differed in their effects on skeletal and cardiac muscle function and chemistry. Perhaps reflecting this, the effects of exhaustive physical exercise influenced the course of infectious illnesses differently, depending on the infection.

Presentations:

1. Brennecke, L. H. and H. A. Neufeld. Pathologic effects and LD_{50} doses of T-2 toxin in rats by intramuscular, subcutaneous, and intraperitoneal routes of administration. Presented at annual meeting FASEB, New Orleans, LA, 15-23 April 1982 (Fed. Proc. 41: 924, 1982).

2. Bunner, D. L. and H. A. Neufeld. Role of ACTH and glucocorticoids in the inhibition of fasting ketosis caused by infection. Presented at annual meeting FASEB, New Orleans, LA, 15-23 April 1982 (Fed. Proc. 41:404, 1982).

3. Bunner, D. L. Review of small molecular weight toxins. Presented, Global Medicine Lecture to Air Force, Brooks AFB, TX, 6 April 1982.

4. Bunner, D. L. Small molecular weight toxins. Presented, Battlefield Medicine Course, Brooks AFB, TX, 27 January 1982.

5. Bunner, D. L. Trichothecene toxins. Presented, Annual short course on Military and Veterinary Medicine, USAMRIID, 5 April 1982.

6. Hauer, E. C. and J. S. Little. Infection-induced RNA synthesis in isolate nuclei. Presentation Annual meeting FASEB, New Orleans, LA, 15-23 April 1982 (Fed. Proc. 41:945, 1982).

7. Neufeld, H. A., F. Beall, D. L. Bunner, J. G. Pace. The inhibition of ketone body formation by insulin in the perfused liver of the fasted rat. Presented, Annual meeting FASEB, New Orleans, LA, 15-23 April 1982 (Fed. Proc. 41:404, 1982).

8. Neufeld, H. A. The effect of infectious and inflammatory stress on fat metabolism. Presented, Hood College, Frederick, MD, 5 April 1982; Hagerstown Junior College, Hagerstown, MD, 8 April 1982; Department of Physiology, LA, State University, New Orleans, LA, 19 April 1982.

9. Pace, J. G. and P. E. Murphy,. Effect of T-2 mycotoxin on the respiratory functions of rat liver mitochondria. Presented, Annual meeting Society for Cell Biology, Anaheim, CA, 9-13 November 1981 (J. Cell. Biol. 91:285a, 1981).

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DAOH 0606	82 09 30	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
82 07 20	K. COMPL	U	U		NL	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AR	196
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE *(Precede with Security Classification Code)* (U) Science Base of Rapid Field Tests for Identification and Diagnosis of BW Agents/Diseases

12. SUBJECT AREAS
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	82 09	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	3.8	137
c. TYPE	d. AMOUNT	83	0.0	0
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Physical Sciences Division, USAMRIID
b. ADDRESS *(include zip code)* Fort Detrick, MD 2170-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR O'Brien, J
d. TELEPHONE NUMBER *(include area code)* 301-663-2833	d. TELEPHONE NUMBER *(include area code)* 301-663-7181
21. GENERAL USE FIC MILITARY/CIVILIAN APPLICATION:	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* Fontelo, R g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Infectious Diseases; (U) BW Defense; (U) Military Medicine; (U) Identification; (U) Diagnosis

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) To perform basic studies concerned with the identification and diagnosis of potential BW agents/diseases/toxemias. The research will be directed toward generating a base of information from which; it will be possible to identify a potential BW agent or toxin within 2-8 hrs from a field sampler with as few as 100-1000 organisms and to diagnose a disease early in the infectious or toxemic process, even before "classic" symptoms become apparent. The achievement of these objectives would substantially aid U.S. Troops to counter act a BW attack by hostile forces.

24. (U) Experimental approaches are following these general lines: immunologic technology concentrates on RIA and ELISA assays, particulary as they relate to recent advances in specific monoclonal antibody production, and/or to the generation of high titered, high affinity of polyclonal antibodies. Bioassay technology concentrates on chemiluminescence of white blood cells and alteration of luminescence of bacteria.

25. (U) 8110 - 8209 - Initial efforts have concentrated on the chemiluminescent (CL) rewsponse of polymochonuclear (PMN) leukocytes to *F. tularensis* and *B. anthracis*. the sensitivity of current CL technology is the detection of 100,000 bacteria. Refinement of the technology should permit the detection of 25,000 bacteria. For internal administrative purposes, these studies are ongoing and will be expanded; however, the work will be reported under a new work unit to be establise in the new fiscal year as 870-BD-074. The chemiluminescent (CL) response of polymorphonuclear leukocytes (PMN) was measured using various species of experimental animals as well as human leukocytes. The Sterne strain of B. anthracis, when opsonified with specific antibody, could detect as few as 100,000 bacteria. Under identical conditions, but opsinifying with a different antisera, F. tularensis could also be detected with 100,000 organisms. The limit of sensitivity of this method is determined by the number of PMN's to produce detectable CL.

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)

Work Unit No. S10 AR 196: Science Base for Development of Rapid Field Tests for Identification and Diagnosis of BW Agents/Diseases

Background:

The project was taken over by MAJ V. O'Brien in December 1981 with the goal of studying the chemiluminescent (CL) response of polymorphonuclear leukocytes (PMN). The CL phenomena is initiated by the binding of an opsonified ligand to a receptor site on the membrane of a PMN, is correlated to the respiratory burst, and is the result of the production of reactive oxygen compounds ($O_2^{\cdot}$, $^{\cdot}OH$, H_2O_2) (1-3). When these compounds oxidize either a bacteria or an artificial substrate (luminol), light is produced in a process termed chemiluminescence (4, 5).

The CL response of PMNs lends itself to studies for the detection and quantitation of those factors involved in particulate recognition, i.e. antigens, antibodies, and complement (6-8). This report reviews studies to quantitate F. tularensis and B. anthracis, and to determine the opsonic requirements for PMN recognition of B. anthracis. Studies are also included to evaluate the effects of trichothecenes on light output by P. phosphorium.

Progress:

The chemiluminescent (CL) response of polymorphonuclear leukocytes (PMN) is measure with the photomultiplier tubes in a liquid scintillation counter (Packard tri-carb) modified to count out-of-coincidence (single photon monitoring). Plastic counting vials containing 2×10^4 PMN in a final volume of 2.0 ml barbital-vernel buffer with 0.5 M luminol (5) are counted for 0.2 min at intervals of between 7 to 24 minutes, depending on the number of samples in the experiment. The data are collected as intensity of CL, a rate function in counts per 0.2 min., vs time in minutes.

Studies on the comparative CL response of PMN from various species challenged with a standard particulate inducer revealed that the greatest response occurred in cells of the human and guinea pig and the least in cells of the rat. Based on the intense response, human PMNs were chosen for our preliminary studies in antigen detection. We found that the Sterne strain of B. anthracis, when opsonified with specific antibody, could easily be detected at 100,000 CFUs. Under identical conditions, only opsonifying with a different antisera, F. tularensis could also be detected at 100,000 CFUs.

The limit of sensitivity of this method is determined by the number of PMNs required to produce detectable CL. We have performed experiments to increase sensitivity but the 1966 Packard appears to be already at its limits of detection. A new instrument has been ordered with special low electrical noise photomultiplier tubes that should allow us to go to a 5×10^3 PMN per vial. This level of sensitivity has been reported by one laboratory and should allow for detection of around 25,000 CFUs of bacteria.

The use of the PMN CL response to detect antigens appears at present to offer little applicability to "field" use, although unforeseen developments could change such a viewpoint. A present advantage that is offered by this technique is time. The assay takes only a few hours, most of that for the isolation of PMNs. Our studies using whole blood directly offer the possibility of a 30 minute test.

Particle recognition by a PMN requires that the particle be opsonified or bound by specific proteins, either antibodies or alternative pathway complement. We used the PMN CL response to determine the opsonification requirements of the Sterne and Vollum strains of B. anthracis.

We prepared irradiated broth cultures of Vollum and Sterne strains of B. anthracis and characterized them by CFUs and protein concentration. Equal CFUs of each strain were opsonified with either normal sera (alternate complement pathway) or with anticell wall sera, treated at 56° (antibody). A very dramatic difference existed between Sterne and Vollum, with the vollum not being opsonified by either alternate pathway complement or antibodies. When Vollum was grown under conditions where it did not have a capsule, it was opsonified the same as Sterne. The capsule of Vollum appears to block opsonification by both the alternate pathway and specific antibody.

The need to detect hazardous compounds in various samples is a matter of concern in the civilian community. Unless a known compound is being sought by a specific assay, general toxicity can sometimes be determined by biologic assays using trout, minnows or microorganisms. Beckman Instruments, Inc. has marketed a "Microtox" kit based on the effect of toxins on the light output of the luminescent bacteria, P. phosphorium.

We have done preliminary studies with P. phosphorium using a liquid scintillation counter to detect light production and found T_2 caused only a 25% reduction in light at 1mg/ml. While these preliminary results indicate that the test is not particularly sensitive for trichothecenes, there is still the potential that it can be used for complex mixtures. While no one toxin may be at a detectable concentration, the combined effect may be detectable. We area studying the additional effects of trichothecenes and will be studying mixtures and crude preparations of molds.

Significant Achievements:

a. Standardized the CL procedures for studies with human, rat and guinea pig PMNs.

b. Determined that the CL response of 2×10^4 human PMNs can readily detect 100,000 CFU of F. tularensis (LVS) and B. anthracis, Sterne strain.

c. Developed a whole blood granulocyte CL assay with rat blood.

d. Determined that the Vollum strain of B. anthracis is not opsonified by alternate pathway complement and that the capsule blocks opsonification by cell wall specific antibodies.

e. Found that changes in the light output from P. phosphorium is not a sensitive indicator of T-2.

Publications:

None

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DAOG 1536	82 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
81 10 01	D. CHANGE	U	U		NL	

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A870	BA	070
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE *(Precede with Security Classification Code)* (U) Risk Assessment and Evaluation of Viral Agents and Their Vectors That Pose a Potential BW Threat

12. SUBJECT AREAS
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	12.0	544
c. TYPE	d. AMOUNT	83	10.0	403
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS *(include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR Bailey, C L
d. TELEPHONE NUMBER *(include area code)* 301-663-2833	d. TELEPHONE NUMBER *(include area code)* 301-663-7241
21. GENERAL USE FIC MILITARY/CIVILIAN APPLICATION:	f. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Hoch, A L g. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Gargan, T P

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Viral Diseases; (U) Arthropod Transmission; (U) Entomology

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Identify specific arthropods and vertebrates associated with the maintenance and transmission of medically important arboviruses to the soldier and define ecologic and intrinsic factors influencing the ability of arthropods to transmit viruses. Information will allow for development of methods for predicting relative risk of military personnel to arbovirus infections and for designing specific vector control strategies.
24. (U) Ecologic and intrinsic factors relating to an arthropod's vector competence to transmit viruses are studied under natural and controlled environments.
25. (U) 8110 - 8209 - A significant correlation was found between the number of laboratory generations of Egyptian strains of *Culex pipiens* mosquitoes and their ability to become infected with and to transmit Rift Valley Fever (RVF) virus. When given a high titer of the virus (10- 6.2 to 10- 7.3 logs), infection and transmission rates averaged 44.5 and 73.8 percent respectively through the first five generations. Between genrations 8 and 12 the infection rates increased while the transmission rates decreased. It was found that transmitting mosquitoes consistently contained more virus which was distributed throughout the body of the mosquito; whereas the virus in infected, but non-transmitting mosquitoes, was lower in titer and limited to the midgut of the insect. A New World species of sandfly, *Lutzomyia longipalpis*, was evaluated as as potential vector of RVF virus. These insects were found to poor vectors of the virus following both oral and intrathoracic routes of infection. A taxonomic key was developed which will allow for accurate identification of culicoides vectors from Kenya. Publications: Am J. Trop. Med. Hyg. 31:15, pp. 1054-1061. Am. J. Trop. Med. Hyg. (In Press).

BODY OF REPORT

Project No. 3M162770A870: Risk Assessment of Military Disease Hazards (U)

Work Unit No. 870-BA-070: Risk Assessment and Evaluation of Viral Agents and Their Vectors That Pose a Potential BW Threat.

Background:

Numerous viral diseases which are normally transmitted by arthropod vectors are potential threats to the mission of US military forces. Some of these diseases are believed to be transmitted in nature only through the bite of an infected arthropod, i.e. Dengue (DEN), Chikungunya (CHIK) and Eastern Equine Encephalitis (EEE) viruses. Others, such as Rift Valley Fever (RVF) virus, can be transmitted not only by an infected arthropod, but also by aerosol. Effective vaccines are not currently available for many of these diseases. Therefore, prevention or control of virus transmission is often achieved only by reduction or avoiding infected vector populations. It is the scope of this work unit to understand the life cycles of arboviral diseases which pose a known or potential threat to military personnel. Included in this scope of research are not only vector and reservoir identification, but also ecological and environmental factors which predispose virus transmission. It is the lack of a clear understanding of these factors that limits our ability to more effectively prevent or control these diseases. RVF virus was first considered to be a serious threat as an arthropod transmitted disease to humans in 1977 when a large epidemic in the Nile Delta of Egypt resulted in considerable human as well as animal mortality. With the above scope and threat in mind the following research objectives were developed: (1) Determine the RVF virus vector potential of several species of mosquitoes and sandflies from Africa, Southeast Asia, South America and the United States which are known to feed on both humans and domestic animals. (2) Determine the effect of environmental temperature and geographic strain of the vector on the transmission of RVF, DEN, CHIK and EEE viruses.

Progress:

Rift Valley Fever Virus

Because the long term colonization of Egyptian Culex pipiens significantly influenced the species competence as a vector, the objective of comparing different geographic strains from Egypt has been deleted from this work unit. Since the effects of long term colonization of the vector on vector competence are not well documented, they will be discussed in this report.

The RVF virus strain used throughout these studies, Zagazig 501, was isolated from human serum in Egypt during 1977. The arthropods for these transmission studies include five strains of Cx. pipiens mosquitoes from Egypt and one strain of Aedes taeniorhynchus from Florida. The sandflies, Lutzomyia longipalpis were originally collected in Brazil. RVF virus infected golden Syrian hamsters were used to infect the insects and non-infected animals were used as transmission hosts. Preliminary experiments showed that the transmission of RVF virus by Cx. pipiens was directly related to the amount of virus ingested with the infectious bloodmeal by the mosquitoes. For example, mosquitoes which initially ingested 10^2 to 10^3 logs of virus had transmission rates ranging from 0 to 10%, while those which ingested 10^4 to $10^{5.4}$ logs of virus had transmission rates of up to 39%.

High transmission rates, approaching 60%, were obtained only when the mosquitoes infective dose was $10^{6.2}$ logs of virus or greater. The purpose of another series of experiments was to determine if sequential generations of Cx. pipiens altered the ability of this mosquito to become infected and to transmit RVF virus; we compared results obtained for 16 generations of the Sharquiya Cx. pipiens strain (Table I). For the purpose of this report, the infection rate is expressed as a percentage based on the proportion of infected mosquitoes. The virus transmission rate, also expressed as a percentage, is the proportion of infected mosquitoes that transmitted virus upon refeeding. There were no significant differences in between transmission rates after 7, 10, and 12 days of extrinsic incubation; therefore, these data were combined. Because of the effect of infectious dose on the mosquitoes ability to transmit virus, transmission experiments were divided into three groups on the basis of the number of plaque forming units (PFU) ingested. The first group received $10^{6.2}$ to $10^{7.3}$ of virus, while the second and third groups received $10^{4.7}$ to $10^{5.6}$ and $10^{2.4}$ to $10^{3.7}$ of virus, respectively.

A trend analysis comparing infection and transmission proportions by the chi-square test of independent proportions was performed on the mosquitoes that received the highest dose of virus.

There were no significant differences between the infection rates from the F_1 through F_8 generation. However, a significant change occurred between the F_8 and the F_{12} when it increased from 78 to 93%, respectively (Chi-square = 27.59, df = 1, $p < 0.005$) (Table I). The infection rates appeared to stabilize following the F_{12} generation.

There were also no significant differences between the transmission rates from the F_1 through F_5 generation. However, a significant change occurred between F_5 and F_8 when transmission decreased from 56 to 25%, respectively (Chi-square = 6.23, df = 1, 0.025, $p < 0.01$) (Table I). Transmission rates did not again change significantly between the F_8 and F_{16} generations.

A comparison of the viral titers for individual transmitting and nontransmitting mosquitoes is presented in Table II. These data suggest that transmitting mosquitoes consistantly contain more virus which is distributed throughout the insects body, whereas, the nontransmitters have less virus which may be limited to a gut infection in the mosquito. The mean titer of transmitting mosquitoes was 2 logs greater than the mean titer of nontransmitters. There was no significant difference between the mean titers of those mosquitoes that refed and transmitted virus ($10^{5.4}$) and those that transmitted by probing ($10^{5.3}$). The significance of the probing transmitters lies with the hypothesis that if this behavior occurs in nature, a single female could transmit virus to several susceptable hosts in an attempt to obtain a bloodmeal.

RVF virus infection and transmission comparison between Egyptian Cx. pipiens and North American Ae. taeniorhynchus are presented in Table III. Both species had similar infection rates on days 7, 10, and 12 postinfectious bloodmeal. However, there were significant differences in transmission rates between the two species. With Ae. taeniorhynchus, transmission rates on day 7 were significantly lower than for days 10 and 12. With Cx. pipiens, there were no significant transmission differences between days 7, 10, and 12 extrinsic incubation. When comparing the two mosquitoes species, Ae. taeniorhynchus required a longer extrinsic incubation and higher virus titer than Cx. pipiens before they transmitted at similar rates.

Even though there is substantial epidemiological and laboratory evidence to incriminate mosquitoes as epizootic vectors of RVF virus, the classification of the virus into the sandfly fever group suggests that sandflies must also be investigated as possible vectors. Since African sandflies were unavailable, we chose a New World species, Lutzomyia longipalpis, for these preliminary studies.

The objectives for these studies were to determine if RVF virus would replicate in sandflies following the intrathoracic (IT) and per os routes of infection and to determine if such females could transmit the virus either vertically or horizontally. As shown in Table IV, the virus titers in inoculated sandflies increased by approximately 2 logs during the first 3 days of extrinsic incubation, then stabilized at this level until day 7 when the study was terminated. When sandflies were infected by the IT inoculation route and then allowed to feed on golden Syrian hamsters, a total of 6 out of 67 transmitted, all after day 4 of extrinsic incubation, for a 9.0% transmission rate (Table V). When these same species of flies were fed a virus bloodmeal from hamsters, the amount of virus in each fly decreased by 3 logs by day 3 postinfectious bloodmeal, Table VI. It is not clear whether the small amounts of virus recovered on days 3-10 represent residual virus from the original viremic bloodmeal or very low rates of virus multiplication in the flies. Regardless of the reason there is nothing to suggest that this species is a potential natural transmitter of RVF Virus. In transovarial transmission studies, 3,375 F_1 progeny resulting from female flies which had taken a RVF virus bloodmeal have been tested for virus (Table VII). None of these offspring were infected, suggesting that transovarial transmission of RVF virus in this species is not likely. In addition to sandflies, Culicoides or biting midges have been incriminated as potential vectors of RVF virus, since the virus was recovered from Culicoides in both Kenya and Nigeria. Because of our limited ability to identify the Culicoides of East Africa and because many species are a threat to potentially transmit several arboviruses to humans as well as domestic animals, a taxonomic key including the 56 known species in Kenya, has been developed which will enhance our ability to identify these potential vectors.

A research program was initiated to study various extrinsic and intrinsic factors which influence the vector efficiency of mosquitoes for arboviruses. Although many mosquito species have been implicated as vectors of CHIK virus, epidemiological evidence suggests that Ae. aegypti is the most important vector. Differential susceptibility between geographic strains of Ae. aegypti has been suggested as a limiting factor in the distribution of the disease. On the basis of the enormous genetic variability among geographic strains of Ae. aegypti, it is likely that the vector competence of this mosquito for CHIK virus would vary between natural populations from different geographic areas. Techniques were developed for evaluating this hypothesis. In preliminary tests, CHIK virus-rhesus monkey blood mixtures, viremic mice, and viremic rhesus monkeys were compared as methods of infecting large numbers of mosquitoes; the virus-rhesus blood mixture method proved superior. In addition, 1-3 day old suckling mice were found to be suitable hosts for transmission studies.

A field ecology study to investigate the possibility that EEE virus is maintained over the winter by transovarial transmission in the mosquito vector, Culiseta melanura was begun. The study area is the Pocomoke Cypress Swamp where EEE virus activity is consistently active during the summer. The preliminary findings have shown that EEE antibody was first detected in sentinel quail that were exposed to field populations of Cu. melanura between 23 June and 7 July 1982. These seroconversions correlated in time and space at approximately 6 weeks after the

initial emergence of Cu. melanura from the overwintering larvae population but 2 weeks prior to peak collections of the species which occurred between 24 and 27 May. This suggests that these mosquitoes took their infective bloodmeal soon after emerging and then transmitted the virus with subsequent feeds, rather than acquiring the virus by transovarial means and transmitting with the initial bloodmeal which would have implied transovarial transmission. A total of 1887 overwintering larvae were collected which have yet to be assayed for EEE virus. However, experimental studies were conducted to determine if infected Cu. melanura are capable of transmitting EEE virus transovarially. A total of 65 blood engorged female Cu. melanura were inoculated with EEE virus during 3 separate experiments (Table VIII). Virus was not recovered from 1530 third and fourth stage larvae which resulted from 29 eggs rafts. All mosquitoes refused to take a second bloodmeal which prevented evaluation of transovarial transmission from the second ovarian cycle.

Presentations:

1. Gargan, T. P., C. L. Bailey, C. L. Crabbs, and L. S. Hutchinson. 1981. Experimental Transmission of Rift Valley Fever Virus by Mosquitoes. Presented, Annu. Mtg. American Society of Tropical Medicine and Hygiene. San Juan, Puerto Rico, 16-20 November.

2. Gargan, T. P., C. L. Bailey, C. L. Crabbs, and L. S. Hutchinson. 1981. Experimental Transmission of Rift Valley Fever Virus by Mosquitoes. Presented to the USAMRIID Professional Staff Conference December 1981.

3. Turell, M. Gargan, T. P., and Bailey, C. L. 1982. Culex pipiens as a vector of Rift Valley Fever Virus. Presented at the annual meeting of the Society for Epidemiologic Research, Cincinnati, Ohio, June 1982.

Publications:

1. Bailey, C. L., M. E. Faran, T. P. Gargan, II, and D. E. Hayes. 1982. Winter Survival of Blood-fed and Nonblood-fed Culex pipiens L. Am. Jour. Trop. Med. Hyg., 31(5), pp 1054:1061.

2. Watts, D. M., J. W. LeDuc, C. L. Bailey, J. M. Dalrymple, and T. P. Gargan, II. 1982. Serological Evidence of Jamestown Canyon and Keystone Virus infection in Vertebrates of the DelMarVa Peninsula. Am. Jour. Trop. Med. Hyg. 31:1245-1251.

TABLE I. INFECTION AND TRANSMISSION RATES FOR 16 GENERATIONS OF CX. PIPIENS WHICH ORIGINATED FROM SHARQIYA, EGYPT.

GENERATION	DOSE* (PFU)	INFECTION %	INFECTION (Inf./Tested)	TRANSMISSION %	TRANSMISSION (Trans./Infected)
F_1	7.2 ± 0.4	67	(39/58)	48	(15/31)
F_2	7.3 ± 0.1	86	(12/14)	14	(1/7)
F_3	7.1 ± 0.4	78	(63/81)	44	(16/36)
F_5	6.2 ± 0.3	70	(19/27)	56	(5/9)
F_8	7.2 ± 0.1	78	(70/90)	25	(13/52)
F_{12}	6.9 ± 0.3	93	(169/181)	18	(16/91)
F_{14}	6.6 ± 0.2	95	(99/104)	24	(12/50)
F_{16}	7.1 ± 0.1	84	(125/149)	21	(15/70)
F_6	5.4 ± 0.1	83	(19/23)	17	(2/12)
F_{12}	5.6 ± 0.2	87	(84/97)	12	(6/49)
F_{14}	5.3 ± 0.1	92	(136/148)	27	(18/66)
F_6	2.4 ± 0.1	51	(26/51)	5	(1/19)
F_{10}	3.7 ± 0.4	57	(54/94)	17	(5/30)

*Titer expressed as $\log_{10}$ PFU/mosquito.

TABLE II. COMPARISON OF RVF VIRAL TITERS IN TRANSMITTING AND NONTRANSMITTING EGYPTIAN CULEX PIPIENS.

x	MIN-MAX*	n
Engorged transmitters		
5.4	4.0-7.1	101
Engorged nontransmitters		
3.1	0.7-5.2	297
Nonengorged transmitters		
5.3	4.6-6.4	21

*Titer expressed as $\log_{10}$ PFU/mosquito.

TABLE III. COMPARISON OF INFECTION, TRANSMISSION RATES AND TITERS IN RVFV ENGORGED EGYPTIAN CX. PIPIENS AND NORTH AMERICAN AE. TAENIORHYNCHUS.

SPECIES/ (DOSE, PFU)[a] Incubation	INFECTION %	INFECTION (Inf/Tested)	TRANSMISSION %	TRANSMISSION (Trans/Inf)	MEAN TITER (n) Trans.	MEAN TITER (n) Nontrans.
Cx. pipiens (6.7 ± .2)						
Day 7	92	(46/50)	20	(6/30)	5.8 (6)	3.1 (24)
Day 10	86	36/42)	35	(9/26)	5.6 (9)	3.3 (17)
Day 12	80	(32/40)	33	(7/21)	5.5 (7)	2.6 (14)
Ae. taeniorhynchus (6.4 ± .3)						
Day 7	98	(48/49)	9	(3/33)	6.5 (3)	3.9 (30)
Day 10	83	(25/30)	30	(7/23)	6.4 (7)	4.7 (47)
Day 12	80	(20/25)	50	(7/14)	5.8 (7)	4.7 (7)

[a]Titer expressed as $\log_{10}$ PFU/mosquito.

TABLE IV. REPLICATION OF RIFT VALLEY FEVER VIRUS IN LUTZOMYIA LONGIPALPIS FOLLOWING INTRATHORACIC INOCULATION.[a]

POSTINOCULATION (Days)	NUMBER SAMPLED	VIRAL TITER[b] Mean	St. Dev.	Range
1	16	2.4	0.40	1.7 - 3.0
2	31	2.9	0.24	2.2 - 3.3
3	41	3.4	0.46	2.0 - 4.2
4	46	3.7	0.49	2.4 - 4.6
5	46	3.8	0.34	2.8 - 4.4
6	40	3.9	0.48	1.9 - 4.4
7	34	4.0	0.35	3.2 - 4.6

[a]Two to three day old sandflies were inoculated IT with a mean of $10^{1.8}$ PFU of RVF virus.
[b]Titer expressed as $\log_{10}$ per sandfly.

TABLE V. TRANSMISSION OF RIFT VALLEY FEVER VIRUS TO HAMSTERS BY *LUTZOMYIA LONGIPALPIS* FOLLOWING INTRATHORACIC INOCULATION.[a]

DAYS POSTINOCULATION OF SANDFLIES[c]	TRANSMISSION TO HAMSTERS[b] No. Pos./No. Tested	No. Neg./No. Tested	VIRUS TITER[c] OF OF SANDFLIES Range	Mean
2 0/6	-	NA[d]	NA	
-	6/6	2.2 - 2.3	2.9	
3 0/7	-	NA	NA	
-	7/7	2.0 - 4.2	3.3	
4 0/7	-	2.4 - 4.2	3.6	
-	7/7			
5 1/6	-	3.6 - 4.0	3.9	
-	5/6	2.8 - 4.4	3.8	
6 2/15	-	3.7 - 4.8	4.4	
-	13/15	1.9 - 5.0	4.2	
7 0/14	-	NA	NA	
-	14/14	2.8 - 5.5	4.5	
8 2/4	-	4.8 - 5.6	5.3	
-	2/4	4.2 - 5.3	4.9	
9 1/8	-	4.5 - 5.1	4.9	
-	7/8	4.1 - 5.2	4.8	
Total 5-9	6/47	-	3.6 - 5.6	4.6
-	41/47	1.9 - 5.5	4.1	

[a]Sandflies inoculated IT with approximately $10^{1.7}$ PFU.
[b]2-5 sandflies blood fed on each hamster.
[c]Titers expressed as $\log_{10}$ PFU/insect.
[d]NA = Not applicable.

TABLE VI. SUSCEPTIBILITY OF LUTZOMYIA LONGIPALPIS TO RIFT VALLEY FEVER VIRUS FOLLOWING FEEDING ON VIREMIC HAMSTERS.[a]

DAYS POST FEEDING[c]	NO. INFECTED/ NO. SAMPLED	%	VIRAL TITER[b] Mean	St. Dev.	Range
1	28/28	100	4.7	0.57	3.5 - 5.7
3	25/42	60	2.0	0.57	1.0 - 3.0
5	34/46	74	1.5	0.71	0.7 - 3.0
7	25/43	58	1.4	0.55	0.7 - 2.8
9	10/46	22	1.9	0.46	1.0 - 2.5
10	3/4	75	0.9	0.21	0.7 - 1.1

[a]Sandflies examined immediately after feeding contained from $10^{4.2}$ to $10^{5.6}$ (mean = $10^{5.1}$) PFU.
[b]Titers expressed as Log_{10} PFU per infected sandfly.

TABLE VII. TRANSOVARIAL TRANSMISSION STUDIES WITH LUTZOMYIA LONGIPALPIS FOLLOWING BLOOD FEEDING ON SEVEN VIREMIC HAMSTERS.

VIRUS TITER OF BLOOD FED FLIES (Day 0)	TOTAL F_1 PROGENY TESTED[b]	NO. OF POSITIVE SANDFLY POOLS
Range (x)[a] 3.4 - 5.0 (4.3)	3375	0

[a]Titers expressed as log_{10} PFU per insect.
[b]F_1 progeny were tested in pools containing from 1 to 50 flies.

TABLE VIII. EVIDENCE AGAINST TRANSOVARIAL TRANSMISSION OF EEE VIRUS BY EXPERIMENTALLY INFECTED CULISETA MELANURA.

DATE	EXPERIMENT NUMBER	NO. OF FEMALES INOCULATED	NO. EGG RAFTS/ DAYS LAID P.I.[a]	NO. POOLS	VIRUS ISOLATION TOTAL LARVAE
5/23/82	1	25	2/3	14	0/140
		6/5			
		4/14	17	0/170	
5/28/82	2	10	2/13	2	0/20
5/29/82	3	30	5/7	51	0/510
		10/9-15	38	0/380	
TOTAL		65	29	122	0/1,530

[a]P.I. = Postinoculation.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DAOG 3814	82 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM
81 10 02	D. CHANGE	U	U			A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A870	BB	069
b. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE *(Precede with Security Classification Code)*
(U) Assessment of Airborne Microbial Agents of Potental BW Threat

12. SUBJECT AREAS
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATE EFFECTIVE — EXPIRATION
b. CONTRACT/GRANT NUMBER
c. TYPE — d. AMOUNT
e. KIND OF AWARD — f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
82	10.2	951
83	21.0	860

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Aerobiology Division, USAMRIID
b. ADDRESS *(include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick MD 2170-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR Stephenson, E W
d. TELEPHONE NUMBER *(include area code)* 301-663-2833	d. TELEPHONE NUMBER *(include area code)* 301-663-7453
21. GENERAL USE FIC MILITARY/CIVILIAN APPLICATION:	f. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Berendt, R F g. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Jemski, J V

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Viral Diseases; (U) Aerosols; (U) Bacterial Diseases; (U) Respiratory Immunity; (U) Lab Animals

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Define aerosol stability characteristics and respiratory infectivity properties of organisms of potential BW importance; elucidate pathogenesis of infections induced by aerosols; evaluate vaccines against aerosol challenge. These studies are used to assess potential BW agents that could be developed by an agressor nation and used against U.S. Forces. The establishment of an organism as a priority potential BW threat permits this Institute to develop appropriate defensive meawsures for use by U.S. Military personnel.
24. (U) Determine aerosol survival characteristics of potential BW agents, develop animal models, including LD-50 information as well as clinical, gross and histopathologic changes during infections. Characterize immune defenses within the reapiratory tract. Use this information to determine the efficacy of vaccination and therapeutic procedures.
25. (U) 8110 - 8209 - Outbred guinea pigs challenged by the respiratory and intraperitoneal (i.p.) routes were exquisitively sensitive to infection with Junin virus. Clinical signs of disease were identical for the two routes of infection. Attenuated VEE virus vaccine provided complete protection, as measured by mortality, against aerosol and i.p. challenge of virulent VEE virus. Inactivated VEE vaccine induced a protective response that varied from partial, to incomplete against an aerosol challenge, while stimulating complete protection against a parenteral inoculation. Passive transfer of humoral antibodies to VEE virus did not impart protection in hamsters. Preliminary data suggest VEE vaccine breaks resulted from virus transversing the cribtiform plate. The attenuated Sterne strain *Bacillus anthracis* spore vaccine induced immune protection in outbred guinea pigs (Hilltop) vs small-particle aerosol challenge; only 50% of those vaccinated with protective antigen-type vaccine were protected. A correlation did not exist between the concentration of humoral antibodies and the survival rate. Publications: Am. Clin. Lab. Sci. 11:452, Infect Immun. 34:766, Am. J. Vet. Res. 43:1429.

BODY OF REPORT

Project No. 3M162770A870: Risk Assessment of Military Disease Hazards (U)

Work Unit No. 760-BB-069: Assessment of Airborne Microbial Agents of Potential BW Threat

Background:

Any microorganism employed as an airborne BW agent against US forces must be stable in aerosols and infective via the respiratory tract. Investigations are designed to define the aerosol stability characteristics and respiratory infectivity potential of possible threat organisms. Additional parameters of the pathogenesis of infections induced by aerosols are elucidated as the need indicates to include determination of the sequence of events leading to immunity following vaccination or aerosol infection. Vaccines developed to protect against potential threat BW agents are evaluated for protection afforded against aerosol challenge. These studies are directed toward identification of efficacious vaccines which will protect deployed troops and at-risk laboratory personnel.

Progress:

Aerosol stability and infectivity evaluations of the Romero strain of Junin virus were extended. A highly significant difference existed at 24°C (75°F) between aerosol stability at 30% RH and stability at 55 and 80% RH. Biological half-life values in aerosol of 27.7, 19.3, and 16.1 min were determined for RH of 30, 55, and 80%, respectively. Outbred guinea pigs were exquisitely sensitive to infection with Junin virus, whether challenged by the respiratory or the intraperitoneal (i.p.) routes. The mean time to death after aerosol exposure, varied from 14 to 18 days and inversely related to the challenge dose of virus, whereas virus concentration exhibited little such dose effects when injected i.p. Differences observed between the median lethal doses for the two routes of challenge were not significant.

Preliminary studies were initiated to define and contrast the pathogenesis of Junin virus infection in guinea pigs induced by aerosol exposure vs i.p. inoculation. One group of outbred guinea pigs was injected i.p. with 500 pfu each of the Romero strain of Junin virus. A second group of guinea pigs was exposed to a small particle aerosol and each animal received an estimated inhaled dose of 350 pfu. Clinical signs of disease were identical for the two routes of infection, except that the fever response generally was evidenced 24 to 48 hr earlier in guinea pigs inoculated i.p. Fever (> 39.0) was detected by day 7 after infection in the i.p. inoculated group and by day 9 in those exposed to a small particle aerosol. The concentration (pfu/g) of virus in the brain, lung, URT, liver, spleen, and kidney of moribund or dead (> 6 hr after death) guinea pigs exceeded $10^{4.0}$ pfu/g for each tissue. These preliminary data indicated that the concentrations of virus attained in the tissues of aerosol exposed guinea pigs were $10^{0.5}$ to $10^{1.0}$ greater than in the tissues of i.p. inoculated animals. Histologically, the Junin-infected guinea pigs could not be segregated according to inoculation route. Each had severe necrosis of bone marrow and extramedullary hemopoietic tissue. The adrenal glands were congested and the lymphoid elements of the spleen, thymus, lymph nodes, and GALT had varying degees

of necrosis. Pathologic lesions were not observed in the central nervous system. Continuing in-depth studies should define the basic pathogenesis of respiratory-induced Junin virus infection in guinea pigs, as contrasted to disease resulting from parenteral injection.

Studies were initiated to evaluate the efficacy of the live, attenuated (TC-83) and the formalin-inactivated (C-84) VEE vaccines vs aerosol challenge. One group of Golden Syrian hamsters was vaccinated with a single dose of TC-83 vaccine, and a second group was given 2 doses of C-84 vaccine. Subsequently, each group was divided into 7 subgroups: antibody evaluation, aerosol challenge/low dose, aerosol challenge/high dose, subcutaneous (s.c.) challenge/low dose, s.c., challenge/high dose, sham aerosol challenge, and sham s.c. challenge.

Estimates of the humoral antibody titers in the challenged hamsters were made by determining the titers in sera obtained on the day of challenge from 10 hamsters of each vaccine group. Geometric mean antibody titers evidenced by TC-83 vaccinated hamsters were 257, as measured by PRN 80, and 955 as determined by RIA. By contrast, hamsters vaccinated with C-84 vaccine had geometric mean humoral antibody titers of 8 by PRN 80 evaluation and 60 by RIA measurement. The Trinidad strain of VEE virus was used for challenge of vaccinated hamsters (Table I). The TC-83 vaccine provided complete protection, as measured by mortality, against challenge doses of $10^{4.72}$ LD_{50} of virus, regardless of exposure route. Inactivated C-84 vaccine, on the other hand, induced a protective response that varied from partial to incomplete. All hamsters exposed to the high dose of virus in aerosol died by day 4 after exposure. Three of the 14 hamsters exposed to the low dose aerosol died, thus indicating the existence of a dose response event. Similarly, three of the hamsters challenged by s.c. injection died. The mean time to death correlated closely to the values obtained for the non-protected control hamsters. The only outstanding exception was one hamster that died on day 10 after exposure to a low dose aerosol.

Concentrations of infectious VEE virus detected in the tissues of non-vaccinated hamsters was essentially the same for both s.c. and aerosol exposures and for each dose of virus. The concentrations varied from $10^{7.0}$ to $10^{8.0}$ pfu/g of tissue. All of the non-vaccinated hamsters had similar lesions, regardless of the route or dose of challenge. Primary changes observed were hemorrhage and necrosis. The most severe hemorrhages noted were in the small and large intestines. Diffuse, severe necrosis was present in the bone marrow, lymph nodes, and GALT (lymphoid tissues) of the small and large intestines. Lesser necrotic alterations were seen in most of the other organs throughout the body. The severe damage to the bone marrow and lymphoid tissues would severely compromise host immune and tissue oxygenation mechanisms, and probably was the primary cause of death.

Since none of the hamsters that were vaccinated with TC-83 attenuated vaccine died, five hamsters from each group were killed on day 5 after challenge. Infectious virus was not recovered from the blood or any of the tissues of the animals challenged by s.c. inoculation or with the low dose aerosol. Surprisingly, low titers of virus approximating 200 pfu/g were detected in the blood, lungs, and brain of hamsters exposed to aerosol containing the high dose of virus. None of the animals examined exhibited significant histopathologic lesions, even in the presence of low grade virus infection. For evaluation of the C-84 vaccinated animals, five hamsters in each of the two s.c. challenge groups and the group exposed to low dose aerosol were killed on day 5 after challenge.

Again, hamsters surviving virulent challenge did not have detectable virus in their blood or tissues, and did not exhibit significant histopathologic lesions. The one hamster that died after challenge s.c. with low dose had minimal to mild necrotic changes. The changes appeared insufficient to cause death, although the concentrations of virus in the tissues mimicked those found in the nonvaccinated control hamsters. Of the two hamsters that died following high dose s.c. challenge, one had lesions analogous to those observed in the non-vaccinated controls which was indicative of a vaccination failure. The second hamster that died in this group had no significant lesions. Interestingly, the virus concentrations in the various tissues were similar in both hamsters. Animals that died after low dose aerosol challenge yielded slightly lower concentrations of virus from their tissues than did the hamsters exposed to high doses of virus. It should be noted that VEE virus replicated to higher titers in the brain, as compared to other tissues. Each of the hamsters that died at both dose levels exhibited similar histopathologic changes. Moderate to severe encephalitis or meningoencephalitis was consistently observed, and was the most likely cause of death. The lesions consisted of varying foci of inflammatory cells, necrosis, and hemorrhage. One hamster had necrosis of the nasal epithelium, which extended through the cribriform plate and into the olfactory lobe. This finding, combined with the higher virus titers in the brain is suggestive of a direct extension of the virus into the brain; however, it does not explain the pathogenesis of the meningoencephalitis.

Additional studies were initiated to evaluate the protective efficacy of passively transferred humoral antibodies to VEE virus vs aerosol challenge. The concept was to transfer high titered antisera obtained from hamsters vaccinated with TC-83 vaccine and challenged with virulent virus via i.p. injection into naive hamsters to attain circulating antibody titers of 200 or greater, then challenge the recipients with a small particle aerosol. Preliminary data indicated that humoral antibodies alone received by passive transfer did not impart protection in hamsters. The mean time to death was not significantly different from that observed for non-protected control hamsters.

Previously reported data indicated that sublethal doses of influenza virus followed by sublethal doses of Legionella pneumophila led to death (1), which seemed to be due to proliferation of the virus rather than of the bacterium. This observation suggested that a "toxin" may have been produced by L. pneumophila and may have caused death. Organisms in a heavy suspension (about 10^9/ml) of L. pneumophila in tryptose-saline were lysed with ultrasonic treatment. Soluble fractions (supernatant fluid) and cell debris (sediment) were collected by centrifugation. The debris was suspended in the original volume of fluid. Fifteen AKR/J mice were given an intranasal sublethal dose of influenza virus, followed 3 days later by an intranasal dose of the crude supernatant fluid. Fifteen mice were treated similarly, but were given the debris that had been collected by centrifugation. Controls consisted of mice given influenza virus followed by saline at 3 days. The data are given in Table 2. Both debris and supernatant fluid significantly exacerbated the influenza infection. This experiment strongly supports the hypothesis of toxic activity.

A number of experiments have been performed to study the transmissibility of L. pneumophila from infected to normal guinea pigs. When infected guinea pigs were housed with normal animals, no evidence of transmission was found (1). Since legionellosis in man and guinea pigs is manifested as a pneumonia, the lack of transmission is difficult to explain. Therefore, investigation of animal to animal passage was initiated. One possibility was that the number of cells needed to infect was so great that insufficient numbers were shed to achieve it. Therefore, the median infective dose (ID50) was reevaluated (Table III).

Groups of 8 guinea pigs each were given graded aerosol doses ranging from $10^{2.23}$ to $10^{5.23}$. Temperature and weight were measured daily. Serum micro-agglutination (MA) titers were determined on blood obtained at 14 and 21 days. The ID_{50} based upon weight loss (5%) and fever (>103.5°F) was about 300 cfu. Based upon serology the ID_{50} was <170 cfu, and by extrapolation was probably close to 17 cfu. An interesting observation was that three of the guinea pigs that did not manifest clinical signs of illness were infected, as shown by sero-conversion. Moreover, when the guinea pigs were rechallenged with 10^6 cfu at 28 days, the only guinea pig that responded was the single animal with no MA titer. In view of the relatively low ID_{50}, the possibility was considered that normal guinea pigs inhaled the equivalent of an infective dose; however, the time required to accumulate the infective dose was too long to establish infection, i.e., normal clearance mechanisms were able to clear inhaled organisms before they could establish infection.

In separate experiments, attempts were made to determine whether organisms could be recovered from the air surrounding infected guinea pigs, from their fur, or from the trachea of normal cagemates. Air samples were taken from the cross-infection box (1) using AGI samplers twice daily for 30 min each. No organisms were recovered when these samples were spread on CYE agar, nor did the acid treatment of Bopp et al (2) aid in recovery. For fluorescence microscopy, a 20 ml sample was recovered from air, centrifuged at 29,000 x g for 5 min, resuspended in 0.5 ml of water, smeared on slides and strained with specific conjugate. The slides were then examined with a fluorescence microscope. Small numbers of fluorescing bacteria were seen in samples taken more than 48 hr after exposure. It is possible, however, that these organisms originated from the fur of the infected guinea pigs.

To determine coat contamination guinea pigs were exposed to an aerosol dose of 10^6 cfu, and samples of the back and head were obtained with moistened swabs at periodic intervals. Large numbers of Legionella were recovered for 5 hrs, lesser numbers for 24, and a trace at 48 hrs (Table IV). This observation suggested that some bacteria are shed following whole body exposure, but it does not indicate whether organisms are shed from the lungs.

A last attempt to determine whether normal guinea pigs inhaled organisms shed by infected cagemates dealt with isolation from the trachea. The rationale here was that the turbulence in inspired air caused by the restriction of the airway as it passes though the larynx should cause deposition of organisms in the trachea just below the larynx (Martonen, personal communication). Accordingly, 4 guinea pigs were placed with infected cagemates. At 3 and 4 days two animals were killed and their tracheas removed. The tissue was homogenized in a small volume of tryptose-saline and spread on CYE plates. The counts, shown in Table V, unequivocally demonstrate that guinea pigs do inhale organisms from infected cagemates. The reason why this does not result in seroconversion or clinical illness is not clear.

Previously reported data shows that > 80% of Michigan Department of Public Health (MDPH)-vaccinated guinea pigs were protected against an intra-muscular (i.m.) challenge containing 100-200 LD_{50}'s (5,000-10,000 spores) of virulent Bacillus anthracis, Vollum 1B (1). These results are in agreement with data published from 1960 to 1970 when the MDPH was developed. Surprisingly, no data are available on the efficacy of the MDPH vaccine to protect guinea pigs against an aerosol challenge of anthrax spores. Studies were implemented, therefore, to evaluate this aspect of vaccine efficacy. Outbred Hartley strain guinea pigs were vaccinated with the MDPH vaccine using the standard regimen of 3 s.c. injections of 0.5 ml each given at

2-week intervals. The presented aerosol dose was calculated to be 152,000 spores of anthrax (aerosol LD_{50} for guinea pigs averages 60,000 spores). All of the saline-vaccinated control guinea pigs died by 4.5 days, with a mean time of 3.5. The survival rate for the vaccinated guinea pigs was 50% and the time-to-death ranged from 3 to 7 days. The geometric mean serum titer of the vaccinated guinea pigs was 832, as measured by the indirect hemagglutination assay (IHA). Serum titers ranged from < 2 in two animals to 4096. A correlation between antibody concentration and guinea pig death or survival was not observed. The survival response data obtained in these initial tests suggested that the protective antigen type vaccine (MDPH) may not be as protective against aerosol challenges as against i.m. challenge.

In all subsequent studies, an additional group of guinea pigs injected with the live, attenuated spore Sterne vaccine was included for contrast evaluation. The spore vaccine ($10^{6.0}$ spores/ml) was administered as 0.2 ml s.c. for the 1st injection, 0.4 ml s.c. for the 2nd injection, and 0.5 ml s.c. for the 3rd injection. Injections were at 2-week intervals. Two weeks after the 3rd vaccination, half of each group of guinea pigs were challenged by aerosol exposure and the remaining animals by i.m. injection with virulent anthrax spores. At 24 hrs prior to challenge, 8 guinea pigs from each vaccinated group and 2 animals from each control group were exsanguinated and the serum was assayed for humoral antibodies using the IHA and ELISA procedures. In both assays, the PA component was used as the antigen. All of the control guinea pigs exposed to either type of challenge died within the usual 2 to 4 day post-challenge period (Table VI). All Sterne spore-vaccinated guinea pigs survived both the aerosol and i.m. challenges. The MDPH-vaccinated animals also were adequately protected (88% survival) against the i.m. challenge. However, when exposed to aerosols of anthrax spores, only 38% of the MDPH-vaccinated guinea pigs survived. Again, no apparent correlation was observed between the concentration of serum antibodies and survival rate. Differences in antibody titer values demonstrated by the two assay techniques reflect an increased sensitivity of the ELISA procedure.

Passive immunity studies were conducted to evaluate the significance of humoral antibodies induced by the MDPH and Sterne vaccines in providing resistance against lethal anthrax. Anthrax immune serum was derived from donor outbred Hartley strain guinea pigs vaccinated with either MDPH or Sterne spore vaccine using the 3 dose regimen. Control guinea pigs were sham-vaccinated with saline. Sera were obtained from donor and control guinea pigs two weeks after the last vaccine injection. All sera were inactivated at 56°C for 30 min before inoculation into recipients. Titers of the donor sera were measured using the IHA and ELISA procedures (Table VII). Blood samples for serum were obtained from all recipient guinea pigs via cardiac puncture at 30 hrs after passive transfer, and the animals challenged shortly thereafter. The inhaled aerosol challenge dose was $10^{5.9}$ and the i.m. challenge dose was $10^{3.65}$ spores. Pre-challenged donor serum showed high titers of humoral antibodies, whereas, marginal or no serum antibody titers were detected for any of the recipient guinea pigs just prior to challenge. None of the control guinea pigs that received normal guinea pig serum survived virulent challenge. Similar results were obtained in a repeat study. Only one guinea pig within both groups of passively vaccinated animals (MDPH vs aerosol challenge) survived the anthrax spore challenges. The role of humoral antibodies in anthrax resistance, however, will remain uncertain until anthrax antiserum can be appropriately transferred to recipient animals to yield measurable pre-challenge serum titers.

State-of-the-art electrophoretic separation techniques and monoclonal antibody production procedures were established. Initial studies have resulted in efficacious subunit vaccine against Coxiella burnetii.

Publications:

1. Lowry, B. S., R. F. Berendt, and K. W. Hedlund. 1981. Early tissue invasion by Legionella pneumophila following aerosolization into guinea pigs. Ann. Clin. and Lab. Sci. 11:452.

2. Jemski, J. V. 1981. Respiratory tularemia: Comparison of selected routes of vaccination in Fischer 344 rat. Infect. Immun. 34:766-772.

3. Kelly, D. J., J. V. Osterman, and E. H. Stephenson. 1982. Rocky Mountain spotted fever in areas of high and low prevalence: Survey of canine antibodies to spotted fever rickettsiae. Am. J. Vet Res. 43:1429-1431.

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2. Bopp, C. A., J. W. Sumner, G. R. Moris, and J. G. Wells. 1981. Isolation of Legionella spp. from environmental water samples by low pH treatment and use of a selective medium. J. Clin. Microbiol. 8:329-338.

TABLE I. PROTECTIVE EFFICACY IN HAMSTERS OF LIVE, ATTENUATED (TC-83) AND FORMALIN INACTIVATED (C-84) VEE VACCINES VS AEROSOL (A) AND SUBCUTANEOUS (SC) CHALLENGE WITH TRINIDAD STRAIN OF VEE VIRUS

VACCINE	CHALLENGE		DEAD/TOTAL (%)	MDTD[a] (range)
	Route	Log_{10} pfu		
TC-83	A	2.55	0/14 (0)	-
TC-83	A	4.72	0/14 (0)	-
TC-83	SC	2.93	0/14 (0)	-
TC-83	SC	5.23	0/14 (0)	-
C-84	A	2.55	3/14 (21)	7.0 (4-10)
C-84	A	4.72	14/14 (100)	4.0 (4)
C-84	SC	2.93	1/14 (7)	4.0 (4)
C-84	SC	5.23	2/14 (14)	5.5 (5- 6)

[a]Mean days to death, geometric mean

TABLE II. EFFECT OF SONICALLY DISRUPTED L. PNEUMOPHILA PREPARATIONS ON INFLUENZA INFECTIONS IN AKR MICE

TREATMENT ON INDICATED DAY		DEAD/TOTAL
-3	0	
Influenza	Saline	6/15
Influenza	Legionella supernatant	13/15[a]
Influenza	Legionella debris	13/15
Saline	Legionella supernatant	1/15
Saline	Legionella debris	1/15

[a]This value is significantly different than the influenza control, $P < 0.01$ by Fishers exact test.

TABLE III. MEDIAN INFECTIOUS DOSE OF L. PNEUMOPHILA IN GUINEA PIGS

DOSE INHALED	NO. POSITIVE/TOTAL		
	FEVER[a]	WEIGHT-LOSS[b]	SEROLOGY
1.7×10^2	3/8	4/8	7/8
$\times 10^3$	7/8	5/8	8/8
$\times 10^4$	8/8	6/8	8/8
$\times 10^5$	8/8	8/8	8/8
ID_{50}	280 (83-847)[c]	310 (21-4607)	170

[a] 103.5F
[b] 5%
[c] Figures in parentheses are 95% confidence limits

TABLE IV. ISOLATION OF L. PNEUMOPHILA FROM THE FUR OF EXPOSED GUINEA PIGS

SITE	MEAN ISOLATION SCORE AT INDICATED HRS[a,b]						
	0.25	1	3	6	24	48	72
Head	+3	+3	+3	+3	+2	+1	0
Back	+2	+2	+2	+2	+2	1	0

[a]Score: +3 =>100 cfu; +2 = 50-100; +1 = <50
[b]Each value is the mean of 4 determinations

TABLE V. ISOLATION OF L. PNEUMOPHILA FROM THE TRACHEA OF CAGEMATES OF INFECTED GUINEA PIGS

DAY	ISOLATION SCORE[a]
3	+1
4	+2.5

[a]Score +1 = <50 cfu/plate; +2 = 50-100; +3 = >100

TABLE VI. RESPONSE OF VACCINATED GUINEA PIGS TO ANTHRAX SPORE CHALLENGE

VACCINE	SERUM TITER[a] IHA	SERUM TITER[a] ELISA	IM CHALLENGE ($10^{3.7}$) SURVIVORS TOTAL	IM CHALLENGE ($10^{3.7}$) SURVIVORS %	AEROSOL CHALLENGE ($10^{6.6}$) SURVIVORS TOTAL	AEROSOL CHALLENGE ($10^{6.6}$) SURVIVORS %
Sterne (spore)	97	1,100	7/7	100	8/8	100
MDPH	558	2,878	7/8[b]	88	3/8[b]	38
Control (saline)	0	0	0/4	0	0/3	0
Control (room)	0	0	0/3	0	0/3	0

[a]Reciprocal geometric mean serum titer
[b]No statistically significant (x^2) from each other at $P < 0.05$, but significant at $P < 0.10$

TABLE VII. RESPONSE OF PASSIVELY VACCINATED GUINEA PIGS TO ANTHRAX CHALLENGE

	SERUM TITER IHA PRE[a]	SERUM TITER IHA POST[b]	SERUM TITER ELISA PRE	SERUM TITER ELISA POST	AEROSOL CHALLENGE ($10^{5.9}$) S/T[c]	IM CHALLENGE ($10^{3.65}$) S/T
Control[d]	0	0	0	0	0/8	0/10
MDPH	4,096	2	77,750	125	1/8	0/12
Sterne	2,576	2	1,846	125	0/8	0/11

[a]Pre - titer of donor immune serum before passive transfer
[b]Post = titer of serum in recipient animals at 20 hrs after passive transfer
[c]S/T = number survivors/number challenged
[d]Normal guinea pig serum

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
				DAOG 3811	82 10 01	
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
81 10 01	D. CHANGE	U	U		NL	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A870	BC	068
b. ~~CONTRIBUTING~~				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE *(Precede with Security Classification Code)*
(U) Technology Development for Rapid Detection and Identification of BW Agents

12. SUBJECT AREAS
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	7.9	413
c. TYPE	d. AMOUNT	83	8.0	329
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Pathology Division, USAMRIID
b. ADDRESS *(include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR LeDuc, J W
d. TELEPHONE NUMBER *(include area code)* 301-663-2833	d. TELEPHONE NUMBER *(include area code)* 301-663-7655
21. GENERAL USE FIC MILITARY/CIVILIAN APPLICATION:	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)* g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Viral Diseases; (U) Bacterial Diseases; (U) Immunology

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Develop assay procedures to rapidly identify agents of BW and special geographic importance. This information will be used to improve the defense ability of U.S. Forces to conduct operations in a BW environment.
24. (U) The enzyme-linked immunosorbent assay (ELISA) is the accepted technology. Initial studies have concentrated on the detection of antigen and early antibody in Rift Valley fever (RVF) and Venezuelan equine encephalitis (VEE) viruses.
25. (U) 8110 - 8209 - Standardized procedures allow complete testing of either RVF or VEE antigens within 5-7 hours. Both polyclonal and monoclonal antibodies were examined as either capture or secondary antibodies for the RVF ELISA. Results suggest that polyclonal antisera are generally more sensitive than monoclonal antibodies as capture antibodies. Under optimal conditions, the ELISA can detect to a 10-5 dilution of RVF antigen; however, the exact antigenetic mass which this represents has yet to be determined. Investigation of pathogens of special geographic importance was concentrated on hemorrhagic fevers with renal syndrome, especially Hantaan virus, causative agent of Korean hemorrhagic fever. The specificity of immunofluorescent assays for rapid detection of both antigen and antibody to Hantaan virus was confirmed by plaque reduction and neutralization tests. Because Hantaan virus has been detected in both laboratory and wild urban rats in major Asian urban centers and associated human disease has been documented, we investigated the possibility that Hantaan virus may have been diseminated through international shipping. Studies have examined sera from wharf rats captured at various OCONUS localities and found positive individuals at several world-wide locations.

Body of Report

Project No. 3M162770A870: Risk Assessment of Military Disease Hazards (U)

Work Unit No. 870 BC 068: Technology Development for Rapid Detection and Identification of BW Agents

Background:

USAMRIID has been tasked with the development of assay procedures to rapidly identify microorganisms or toxins which are potential biological warfare (BW) threats or which have special geographic importance. The goal is for same-day identification of agents. The unified (common) technological approach selected for development is the enzyme linked immunosorbant assay (ELISA). Anticipated antigenic material to be identified will come from either collector fluids of the XM-2 Collector or from clinical samples from acutely ill patients. Due to the particle density required to produce effective aerosol transmission, antigen should be relatively abundant in collector fluid samples, while antigen will be of variable but generally lower concentration in clinical specimens.

Agents of concern as potential BW threats against US Forces are as follows, Bacteria and Rickettsia: Anthrax[2]; Tularemia[2]; Brucella[3]; Plague[1]; and Q fever[2]; Viruses: Venezuelan equine encephalitis (VEE)[1]; Rift Valley fever (RVF)[1]; Tick-borne encephalitis (TBE)[2]; Lassa[1]; Machupo[3] (=Bolivian hemorrhagic fever); Junin[1] (=Argentine hemorrhagic fever); Marburg[3]; Ebola[3]; and hemorrhagic fevers with renal syndrome (HFRS); Toxins: Botulinum[2]; marine toxins[3]; Staph. enterotoxin[3]; and mycotoxins[2]. Monoclonal antibodies have been developed for all agents marked by [1]; monoclones are in the process of being developed for those marked by [2]; and monoclonal antibody studies will soon begin for those marked by [3].

Agents of primary geographic concern are Bacteria: Shigella, Salmonella and plague; Toxins: E. coli; Rickettsia: R. conorii; Viruses: Rotaviruses; Dengue viruses; RVF; Sandfly fever group viruses; Congo-Crimean hemorrhagic fever; West Nile; HFRS and Chikungunya viruses, and Parasites: Malaria and schistosomiasis.

The basis for the selection of the ELISA as the unified technique of choice for rapid identification of multiple agents was: (A) the relative ease of operation; (B) simplicity of equipment needed for the assay; (C) the relatively good sensitivity of the system (detection limit approximately 0.01 ng/ml in either saline collector fluid or human serum); (D) the potential for extreme specificity through incorporation of monoclonal antibodies in the testing procedures; and the ability to identify diverse kinds of single agents using a final common readout technique with a single standardized instrument.

Epidemiological investigations have been initiated on selected agents of special geographic importance to better understand the potential impact of these agents on military operations and to provide relevant clinical samples for testing in developed rapid diagnostic assays. HFRS and certain viruses endemic in the Middle East are being investigated. HFRS study samples were obtained by our field team or supplied by OCONUS collaborators. Samples from the Middle East have primarily been obtained from US military forces deployed to that area.

Progress:

Studies have been directed towards the development of an ELISA capability for the testing of antigens (AG), and polyclonal, monoclonal, and affinity-purified antibodies of RVF virus. (4) Reagents used in these studies were: AG's hemagglutination-inhibition (HAI), Salk Lot 1-78 and Salk human vaccine TSI-GSD-200 Lot 6 Run 2; antibodies, Salk-produced rabbit (mouse liver AG), guinea pig (FRhL AG), mono- or polyclonal mouse (MoAG) or affinity purified polyclonal mouse serum. Goat anti Mo, guinea pig (GP), or rabbit (Rb) labeled with alkaline phosphatase was used as AB_3, and p-nitrophenylphosphate as substrate. Specific activity was read as optical density at 405 nm. All antibodies were used in a 4-square system in which each was tested against all heterologous species as either capture (AB_1) or secondary (AB_2) antibody. Results are given as the log dilution of the HA antigen detectable by specific sera. Using a constant dilution of GP serum as AB_1, polyclonal mouse AB_2 could detect a 10^{-4} AG dilution, whereas 2/13 monoclones (one of nucleocapsid specificity) could only detect a 10^{-3} AG dilution. When Rb Serum was used as AB_1, results were similar but the AG detection limits of all AB_2 were 1 log less in all studies. Affinity purified AB was not active at the minimum AG dilution (10^{-2}).

Reversing the conditions, i.e., using monoclones as AB_1 and Rb or GP as AB_2 gave similar results with the following observations. Polyclonal mouse was again superior, detecting a 10^{-4} dilution whereas 3/13 monoclones (2 of nucleocapsid specificity) could detect a 10^{-3} AG dilution. Three additional monoclones, 2 of glycoprotein specificity plus affinity purified polyclonal mouse could detect a 10^{-2} AG dilution.

In summary, when polyclonal, monoclonal and affinity purified mouse sera were used as AB_2, polyclonal was superior, detecting 1 log less (10^{-4} dilution) AG than monoclones in which only 2/13 monoclones were active at the 10^{-3} AG dilution value. When used as capture AB_1, polyclonal AB again detected a 10^{-4} AG dilution, 3/13 monoclones could detect a 10^{-3} AG dilution and an additional 3 monoclones (2 of glycoprotein specificity) and affinity purified polyclonal could detect a 10^{-2} AG dilution. When vaccine was used as antigen, the same sera were generally active but less consistently and at lower AG dilutions, i.e., 10^{-2} or 10^{-3}, a factor possibly related to AG load.

Current studies concern the sensitivity of the ELISA to detect AG diluted in whole human serum, sensitivity of protein A purified antibodies as capture and/or secondary AB, defining the limits of sensitivity of all sera of indicated usefulness, and areas to be discussed under the ancillary studies section of this report.

Antigenic Load. All immunoassays suffer to varying degrees from a lack of complete antigen characterization, i.e., definition of total antigenic load. We attempt characterization by defining purity, viability, protein content, defectiveness, total number of particles, etc, but, in reality, finite characterization eludes us.

As an example, the RVFV vaccine had a preinactivation viable titer of 5.5×10^6 PFU/ml and a total particle count of 8.6×10^9, the latter determined by Dr. J. White by electron microscopy. The titers indicate at least a 1000:1 ratio of particles to PFU. This does not begin to include the the amount of amorphous

material present that could be viral subunits which contribute to the total reactive antigenic load of the preparation. A consensus as to what constitutes antigenic load does not exist and will have to be defined on the practical basis of what reacts antigenically in a given system by a "standard" antigen production preparation. The system used for these studies is the maximum antigen dilution of a given preparation producing an optical density (OD) of x sample - x control + 2sd $\geq$ 0.100. Although usable in most instances, even this "standard" varies as new bottles of antigens and control fluids are opened and deterioration begins. Detailed records for each bottle opened will allow a shelf-life to be determined.

RVFV IgM ELISA. Historically, one sought a retrospective diagnosis of virus infections by demonstrating a 4-fold rise in specific antibodies between acute and convalescent sera. This approach implied a measurement of virus specific IgG. In 1979, a new method was developed for RIA using the "antibody capture principal" that has since been adapted to ELISA for detection of virus specific IgM antibodies. Such antibodies generally develop shortly after infection, peak rapidly, and decline as specific IgG is produced. Detection of specific IgM by antibody capture aids in the detection and identification of specific viral diseases.

Initial studies have determined: 1) Sera, column treated to remove IgG and IgG-IgM complexes is less reactive than untreated sera, presumably due to the 10-20% loss of IgM during purification: 2) RVF HAI AG is superior to purified RVF vaccine at comparable dilutions, no doubt due to the increased antigenic load contained in the HAI AG: 3) the number of false positives is less when using unfractionated sera. Studies have been hampered by the lack of adequate numbers of sera obtained early in infection or shortly after vaccination; however, some samples from volunteers receiving RVFV vaccine on research protocols have been received and are being tested.

Virus Reagent Inactivation: We are tasked not only with the development of ELISAs for various viruses but also with the added proviso that all reagents be nonviable prior to use in laboratories without P-3 containment or introduction into geographic areas free of specific endemic disease. Inactivation is achieved by cobalt-60(^{60}Co) irradiation. Inactivation procedures for RVFV reagents were determined prior to initiation of this project (vaccine, formalin; HAI BPL; sera ^{60}Co).

Studies are in progress to clearly define the inactivation curves from which equations will be derived allowing direct calculations of the amount of radiation required to totally inactivate a given concentration of virus. Such studies, modeled after those of Elliott *et al*[5], will be done for all viruses used in this program.

Chikungunya virus ELISA. Chikungunya virus, strain 181 propagated in DBS-103 cells and corresponding DBS-103 cell control fluids (inactivated by ^{60}Co irradiation) and the following antisera consisting of MAF strain S-27, and monkey strain S-27 were supplied for testing by Dr. Levitt, Virology Division. Monkey serum RG-20 and guinea pig serum GPI-1 were supplied by Dr. Rosato, Medical Division.

Initial testing of each serum as both capture (AB_1) or secondary (AB_2) has not resulted in clearly defining which is optimal in either position, for each reacts to varying degrees with the control cell fluids. We will continue to define the conditions necessary for a usable Chikungunya ELISA test.

Congo Virus ELISA. Congo virus, strain IbAr 10200 supplied by Dr. Luscri, Virology Division as ^{60}Co inactivated suckling mouse brain (SMB) homogenate was tested in a standard ELISA against GP (G-18) and Mo (NIH 3010) sera in which each serum was used as both capture and secondary AB. A ^{60}Co treated normal SMB homogenate was used as an internal control.

In all tests the normal SMB control reacted with equal or greater intensity than the virus containing preparation. The addition of normal GP or Mo serum to all dilutions post AB_2 did not alleviate non-specific reactions. Similar difficulty with SMB preparations of arboviruses used in ELISA development has been reported by Frazier and Shope. Dr. Luscri has been requested to prepare another Congo virus stock propagated in a cell culture system. Testing will begin upon receipt.

VEE ELISA. The detection of VEE antigen by ELISA has now been standardized based on the procedure ofDr. Robert Yolken (Johns Hopkins University).

VEE antigen was detected in the range of 2.8×10^6 pfu/ml. In previous experiments, the same amount of VEE antigen was detected when the AB_1 used was affinity purified rabbit anti-VEE and AB_2 was protein A purified monkey anti-VEE. By using pooled higher titered human anti-VEE as AB_2, the cumbersome purification procedure could be eliminated without sacrificing sensitivity. Even when only 0.05 ml of antigen was in the test instead of the unusual 0.1 ml/well, positive results were obtained at 10^{-3} dilution of the antigen.

Conjugation of rabbit anti-VEE with horse radish peroxidase and using it as AB_2 shortens the procedure by one step but retains the same sensitivity. A similar conjugate prepared by Dr. Yolken detected the same amount of antigen. This conjugate was used in a direct assay and no difference in sensitivity was noted.

In order to stimulate clinical specimens, pooled normal serum was used as diluent for the VEE antigen. The sensitivity of the assay dropped by one log (2.8×10^7 pfu/0.1 ml), probably due to non-specific binding by serum proteins, which increased the background reading. When the collector fluid (KPBS-Tween) was used as antigen diluent, no loss in sensitivity occurred.

The specificity of ELISA was tested by using eastern equine encephalitis and RVFV HA antigens. The former reacted at 1:10 dilution while the latter was completely non-reactive. Other antigens will be tested when they become available.

The shelf life of microtiter plates coated with rabbit anti-VEE when allowed to dry and stored at 4^oC, is approximately 2 months. By day 74, the sensitivity had decreased by 1-2 logs.

There were 5 false positives and 3 false negatives (PRN titers of 1:10, 1:40, and 1:80) when 100 sera (PRN titers known) were screened at 1:20 dilution by ELISA. A review of the histories of the 5 false positives revealed that 2 of the persons had received VEE vaccine many years ago while the remaining 3 had worked or were still working in virus laboratories with no known exposure to VEE. Twenty-four sera were randomly selected from a group of VEE vaccinees who were considered non-responders (no demonstrable antibody by PRN test). Eleven of the 24 sera were positive by ELISA (titers range 1:20 to 1:160) of which 7 were also positive by HAI test (titers range 1:10 to 1:80); 12 sera were negative by both ELISA and HAI assays. One serum was positive by HAI (1:10) but not by ELISA while 4 were positive by ELISA only. These 24 non-responders were originally tested and found negative using the 80% PRN assay. When they were retested using the 50% PRN end point, 15 were positive at 1:10 dilutions. This indicated that the 80% PRN assay missed low titer antibody in VEE vaccinated individuals.

A set of 44 sera with known PRN titers to VEE were titrated by ELISA. There was a good correlation (usually within a 4-fold dilution) between ELISA and PRN when titers were 1:80 or greater; however, when PRN titers were low (1.80), ELISA results were as great as 8 fold higher.

A test for VEE-IgM antibody is being developed. Progress has been delayed by the lack of serially collected sera following VEE immunization and lack of positive controls; however, initial results on sera from two recently immunized individuals are promising.

Ancillary Studies. The standard RVFV ELISA (6) required 3 days for completion, overnight coating of capture AB, and for antigen reaction, 1 h reaction of secondary AB_2 and 1 h each for enzyme-labeled antispecies AB_3 and color development. Attempts to decrease the time required took three approaches. First, the standard 3-day test was compared with reaction times of various durations; second, combinations rather than sequential additions of reactants; and third, the effect of shaking during reactions were examined.

Results show that both overnight reactions (AB_1, AG) at 4C could be decreased to 2 h at 37C and that the AB_2 time of 1 h could possibly be decreased to 30 min. AG time could possibly be reduced further to 1 h. Anti-species AB_3 and color development times were not varied in this series. A "rapid" ELISA for RVF AG is now possible within a working day. Other studies show that the combined addition of AG and AB_2 or AB_2 and AB_3 as a single mixture is possible further decreasing test time 1.5 to 2.0 h. Combining AG, AB_2 and AB_3 as a single mixture does not work. Shaking at 37C on a mini orbital apparatus produces a slight to moderate increase in OD over static conditions.

XM-2 Studies. One of the requirements of these ELISA systems is that they be usable for antigen detection with samples collected by the XM-2 biological field collector, a component of the XM-19 biological field alarm system. Initial studies compared the standard RVF ELISA for AG detection and an ELISA in which standard AG dilution fluid was replaced with XM-2 collection fluid that had been used for ambient sampling for 45 min at 1000 L of air/min. No appreciable differences were seen in the ODs between reactions in standard diluent or XM-2 fluids. Current modifications of the collector fluid, i.e., addition of 0.005 M or 0.0005 M 2-mercaptoethanol (2-ME) are now being tested to determine the effect of 2-ME on the ELISA test for RVF AG.

Epidemiology of hemorrhagic fevers with renal syndrome. Our field team has sampled wharf rat populations at the ports of Philadelphia, Houston, Stockton, Sacramento, Oakland, San Francisco and Los Angeles as part of a serosurvey of Hantaan virus among domestic wharf rats. Rattus sera were obtained at every port sampled with the exception of Stockton. Significant rodent populations were encountered only at the ports of Philadelphia and Houston. All California ports had active rodent control programs in operation and relatively few rats were captured. Rodents were virtually uncontrolled at the ports of Houston and Philadelphia, and more than 100 rats were captured from each of these locations. Sera from all localities have been screened by IFA for anti-Hantaan virus antibody with the following results: Philadelphia, 5 suspect positive of 110 tested (4%); Houston, 12 of 109 (11%); Sacramento, none of 8; Oakland 1 of 5 (20%); San Francisco, 1 of 28 (3%); and Los Angeles, none of 4. Antibody positive rats, when present, appeared to be clustered at a given locality rather than uniformly distributed throughout the greater port area. At Philadelphia, most antibody positive rats were captured at a grain loading operation at Girard Point, while in Houston most were captured at Wharf 14, also a grain loading facility. At Girard Point, 5 of 13 (38%) were suspect positive, while at Wharf 14 in Houston, 5 of 8 (63%) were positive.

A plaque reduction neutralization (PRN) test using the 76-118 strain of Hantaan virus has been developed and 2 of 5 sera from Girard Point and 5 of 5 sera from Wharf 14 in Houston neutralized at $\geq$ 1:20 dilution against approximately 100 plaque forming units of Hantaan virus.

A second visit was made to the Port of Houston during which additional rats were collected from Wharf 14. During this study an additional 6 rats were captured. These were bled and their sera frozen, plus organ tissues from lung, liver, spleen, kidney, and in some, heart and bladder were preserved in formalin, OCT frozen mounting medium and as whole frozen tissue. In addition, urine was saved, or in the cases where the bladder was void, buffered cell culture medium was flushed through the bladder and stored frozen.

Preliminary results are available from the second sample from Wharf 14, Houston. Of the 6 sera tested, 5 had titers to Hantaan virus as follows: 2 at 1:32; 1 at 1:512; and 2 at 1:1024 or greater. When tissues from these animals were examined by direct and indirect IFA using high titered KHF convalescent human serum as a conjugate, the single antibody negative and both sera which titered 1:32 lacked Hantaan virus antigen, but tissues from the rat whose titer was 1:512 and both of the $\geq$ 1:1024 animals had Hantaan virus antigen in their tissues. When lung tissues from these animals were triturated and inoculated intramuscularly into 6 laboratory reared, sero-negative Wistar rats, then screened for development of anti-Hantaan virus antibody 28 days later, 3 of the 6 tissue suspensions inoculated caused the development of anti-Hantaan virus antibody in one or more of the recipient rats. All tissue suspensions which caused seroconversion had been shown previously to contain Hantaan viral antigen. Attempts to isolate a virus from these rats are currently in progress.

A repeat sampling was also made at Girard Point in Philadelphia. Twelve additional rats were captured from that apparent focus of infection, of which 8 had titered ($\geq$ 1:256) IFA antibody to Hantaan virus. When lung tissues were examined for Hantaan viral antigen, 5 were positive, including one from an antibody negative rat. When triturated lung suspensions were inoculated into groups of 6 seronegative

Wistar rats, one or more individuals in each of 5 groups developed anti-Hantaan virus antibody by day 30 post inoculation. Included in those seroconverting were 2 of 6 rats inoculated with tissues from an antigen and antibody negative individual. These results suggest that infectious virus was present in some rats in the absence of either demonstrable Hantaan virus antigen or antibody.

Necropsy tissues from the 12 Philadelphia rats were examined by Drs. Green and Callis of the Pathology Division, USAMRIID, for evidence of Hantaan virus induced pathology. While numerous pathologic conditions were detected, including lung and kidney lesions, nothing was detected which was clearly attributable to past or present Hantaan viral infection.

Our laboratory has also been acting as a reference facility for the study of Hantaan virus in laboratory rat colonies. As such, we have screened over 100 human, rat and other animal sera for anti-Hantaan virus antibody by IFA. Many of our positive IFA results and a sample of our negative IFA results have been confirmed by plaque reduction neutralization tests. Most notable is our ongoing study of Hantaan virus in the rat colonies of the University of Louvain, Belgium. There, several antibody positive people have been identified, some of whom have a clinical history of acute nephritis. A large proportion of rats from certain inbred colonies there also contain anti-Hantaan virus antibody.

We have processed a sample of rat tissues, primarily lungs, from selected rat strains for the detection of Hantaan viral antigen and inoculated sero-negative Wistar rats to look for acquisition of anti-Hantaan virus antibody. All tissues processed were from individual rats which had high-titered IFA anti-Hantaan virus antibody, with the exception of the OKA strain, which titered 1:16. Hantaan antigen was detected in the lungs of 4 rats, strains LOU, LL, LOU/OKA and LM. Seroconversion was detected in rats inoculated with tissues of only 2 strains, LOU and Euro. We plan to process these tissues further in an attempt to recover a cell culture strain of virus.

Recent studies by our laboratory on human and rodent sera collected from various localities in northern Brazil indicate that an agent similar or identical to Hantaan virus is present in that area. Specifically, of 21 Rattus norvegicus collected from urban areas of Belem, 8 had positive IFA reactions ($\geq$ +2 at 1:32 dilution), and 5 of these 8 sera titered $\geq$ 1:2048. A sample of these sera have been examined under code by Dr. Lee of NIH and our positive results were confirmed by him. In addition, Dr. Lee successfully blocked the IFA reaction with reference anti-Hantaan virus antiserum. He also tested these sera by IFA against NE antigen in infected vole lung tissues. Results were either negative or $\leq$ 1:16 on the 2 sera examined.

Human sera from various rural localities in northern Brazil, excluding Belem, have been tested by IFA and several have been found positive, generally in tiers 1:32-1:128. Of 8 sites sampled in Para State totaling 37 sera examined, 4 sites and 7 sera were positive ($\geq$ 1:32 titer; an additional 4 sera had 1:8 reactions). Of 3 sera sampled from 2 sites in Amazonas State, one serum had a 1:32 titer, one 1:8, and one was negative. All 37 sera were previously tested and found to be positive by Dr. Ho Wang Lee in Korea.

We are continuing our investigation of Hantaan virus in Brazil. We have received additional rat sera from foci of infection in Belem; however, we have yet

to examine them. We have also received limited tissue samples for antigen determinations and virus isolation attempts. An attempt to identify human illness caused by Hantaan virus in Brazil is a goal for the coming year.

We have collaborated with Dr. Dave Robinson of the NAMRU-3 laboratory in Egypt in our investigation of Hantaan virus. He has provided 144 rat sera from 3 port localities in Egypt which we have examined by IFA for anti-Hantaan virus antibody. Six sera have been found positive, of which 2 from Port Said were high titered. Both were from a single locality and additional sera from this location are currently being examined. We hope to obtain tissues from rats collected in this area in the near future for virus isolation attempts.

We have received 100 human sera fom sewer workers of Hamburg, Germany. These persons were presumably highly exposed to rats and might be expected to have antibody to Hantaan virus. We have screened all 100 by IFA, but found none reactive of a 1:8 dilution. Attempts to capture rats from Hamburg are now in progress and this study continues.

Dr. Doug Watts of USAMRIID has obtained 101 Rattus norvegious sera from Chincoteague, Virginia, which we have examined for anti-Hantaan virus antibody. Eleven sera were found positive, most from a pig farm. Additional studies in this area are planned.

Sera from rats captured in Rangoon, Burma and Mombasa, Kenya are currently awaiting testing.

Publications:

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2. LeDuc, J. W., A. L. Hoch, F. P. Pinheiro, and A. P. A. Travassos da Rosa. 1982. Enfermedades epidemicas causadas por el virus Oropouche en el norte de Brasil. Bol of Sanit. Panam. 92(2):143-152.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
				DAOG 3810	82 10 01	
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISS'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
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10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	BA	150
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE *(Precede with Security Classification Code)*

(U) Prevention of BW Diseases Caused by Microbial Toxins

12. SUBJECT AREAS

003500 Clinical Medicine; 004900 Defense; 010100 Microbiology; 016800 Toxicology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	10.7	587
c. TYPE	d. AMOUNT	83	10	710
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Pathology Division, USAMRIID
b. ADDRESS *(Include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR Crumrine, M H
d. TELEPHONE NUMBER *(Include area code)* 301-663-2833	d. TELEPHONE NUMBER *(Include area code)* 301-663-7211
21. GENERAL USE FIC MILITARY/CIVILIAN APPLICATION:	f. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Siegel, L S g. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Sellin, L C

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Microbial Toxins; (U) Vaccines; (U) Therapy; (U) Toxoids; (U) Prophylaxis

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Develop and evaluate biologics and selected compounds for prevention and treatment of disease induced by microbial toxins of military importance. Prepare, characterize and produce toxoids/vaccines suitable for protecting U.S. Forces against botulism and anthrax. Collect, produce and test immunoglobulins having efficacy in preventing and treatment of U.S. Forces against microbial toxemias. Develop materials and methods for detection and assay of toxins, to increase "readiness" state of U.S. Military personnel.

24. (U) Develop new technology for fermenter-system production of small experimental lots of microbial toxins and methodology for isolation, purification, alteration and detection of toxins. Immunogenicity of various antigens will be described and converted to experimental toxoids/vaccines. Improve procedures for immuno- and chemotherapy of toxin-mediated diseases will be developed. The action(s) and effects of both toxins and their antagonists will be determined and described at the cellular level.

25. (U) 8110 - 8209 - Conditions for conversion of type A neurotoxin to toxoid were determined. A monovalent type E toxoid from the Michigan Department of Public Health was evaluated in human subjects. Approximately 500L of human-derived botulism immune plasma were converted to botulism immune globulin. Methods to fractionate and despeciate Heptavalent (ABCDEFG) Botulism Immune Plasma (Equine) were evaluated. The relative toxicity of purified types A and E neurotoxins were compared by electrophysiologic measurement. The immunogenicity and efficacy of American and British anthrax vaccines were compared.

Publications: Infect. Immun. 30:381, 1980; Appl. Environ. Microbiol. 40:1023, 1979, 42:1018, 1981; Med. Biol. 59:11, 1981; Curr. Microbiol. 6:127, 1981; chapters in: Natural Toxins, Medical Aspects of Botulism, Staphylococcal Infections.

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)

Work Unit No. 871 BA 150: Prevention of BW Diseases Caused by Microbial Toxins

Background:

Among the bacterial toxins considered to be potential BW agents, the 8 lethal toxins produced by the bacterium Clostridium botulinum and the lethal toxin complex produced by the bacterium Bacillus anthracis are of great concern.

Botulinal neurotoxins (NT) remain the most potent biological toxins known. Their ease of production, stability and lethality assure their position as very high priority toxins for potential use as overt, covert, terrorist and/or BW agents against U.S. military and/or civilian populations. The protection of personnel at risk through exposure to the botulinal neurotoxins, the treatment of exposed individuals and the rapid detection of threat toxins are all of considerable concern to both the field commander and the at-risk laboratory worker. The botulinal toxoid currently in use to induce active immunity in individuals at risk is derived from formalin-inactivated type A, B, C, D, and E toxins. This product was produced by Parke-Davis, under contract to the U.S. Army, in 1958. The Parke-Davis pentavalent toxoid contains only about 10% neurotoxoid and induces sustained measurable antibody titers only after a series of 4 injections administered over a period of 1 year. Mild local reactions, including induration, erythema, and pain at the site of injection, are common. A substantially improved immunogen, prepared from highly purified neurotoxins and capable of inducing a high degree of protective immunity to poisoning by all 8 known types of botulinal toxin, is long overdue. Toward this end, optimal fermentor conditions for the production of type A, B, and E neurotoxin have been developed by USAMRIID scientists (1, 2, 3).

Until recently, the only antitoxin available for the treatment of botulism patients was prepared in horses and provided to the U. S. by a Canadian firm. The equine antitoxin initiates adverse reactions in many recipients and is useful for the neutralization of only 5 (ABCDE) of botulinal neurotoxins.

Treatment with specific antitoxin does not reverse existing botulinal toxin-induced paralysis. However, the compound 3,4-Diaminopyridine (3,4-DAP) is transiently effective in antagonizing the blockage of transmitter release resulting from botulinal poisoning (4, 5). The compound is the pilot drug under investigation as part of an effort to develop a chemotherapeutic approach to the treatment of botulism.

The mouse bioassay methods now available for detecting and assaying, in a variety of media, nanogram quantities of botulinal toxin are both time consuming and cumbersome procedures and require considerable modernization. A conversion to new technology is also overdue, even though the bioassay is extremely sensitive.

Anthrax has long been considered a potential, highly effective BW agent against which USAMRIID must develop improved medical defensive measures. Of particular concern is the development of effective medical countermeasures to the aerosol deployment of anthrax spores. The spores of Bacillus anthracis are extremely stable

under normal conditions and show a high degree of resistance to inactivation by standard decontamination procedures. These characteristics make the use of anthrax spores as a biological warfare agent a threat to be reckoned with. Early clinical signs of pulmonary anthrax are somewhat difficult to interpret, making diagnosis difficult, and once established the clinical illness progresses rapidly. Evenly if rapidly diagnosed there is no consensus of opinion on the optimal treatment. There are 3 distinct components of the anthrax toxin complex: lethal factor (LF), edema factor (EF), and protective antigen (PA). Biological activity has not been described for PA, aside from its immunizing effects. However, PA is required for expression of the biological activity of LF and EF. It is presumed that PA is a major immunogen in the current anthrax vaccine and that antibody to PA is highly effective in preventing anthrax. The currently available anthrax vaccine has several limitations. The immunization schedule for humans consists of 3 initial doses (0.5 ml each) at 0, 2, and 4 weeks followed by boosters at 6, 12, and 18 months; yearly boosters are needed thereafter to maintain immunity (6). The impurity of the anthrax vaccine may contribute to the common occurrence of unpleasant side effects and to the low titers of induced immunity. A more effective anthrax vaccine, one which induces high titers of protective immunity in a relatively short time and after only a few injections, is highly desirable.

Progress:

Numerous studies were conducted by Dr. Lynn Siegel to determine optimal conditions for the formalin type A botulinal neurotoxin. (All U. S. licensed toxoids have been produced with formalin.) All neurotoxin preparations inactivated by 0.6% formalin were rendered nontoxic within 72 hours. To further accelerate toxoiding conditions, type A neurotoxin was incubated with 0.6% formalin in the presence of 0.01 and 0.1 M L-lysine or without lysine. All preparations containing lysine were detoxified within 48 hours and remained nontoxic during 28 days of sampling.

In general, antigens prepared with formalin are more immunogenic when combined with an adjuvant. Therefore, the efficacy of a fluid type A neurotoxoid versus an aluminum hydroxide adsorbed neurotoxoid was evaluated. (Only aluminum compounds are used as adjuvants in toxoids licensed in the U. S.) Type A neuro-toxoid prepared with 0.6 formalin (28 days incubation) was adsorbed to $Al(OH)_3$ (final concentration of $Al(OH)_3$, 4 mg/ml). Mice immunized with various concentrations of fluid or $Al(OH)_3$ prepared toxoid were challenged with one of several toxin doses and the degree of protection provided by each neurotoxoid was determined. Mice injected with fluid toxoid, like those in the control groups, did not survive the lowest challenge dose of crude toxin (1×10^3 LD_{50}). However, mice injected with the $Al(OH)_3$ adsorbed neurotoxoid survived a challenge dose of 2×10^5 LD_{50}.

A preliminary experiment was performed to compare the kinetics of detoxification by formaldehyde (0.6% formalin, equivalent to 0.0739 M formaldehyde) with detoxification by an equimolar concentration of glutaraldehyde. Type A neurotoxin (final concentration, 5.0×10^6 LD_{50}/ml) was rendered completely nontoxic for mice in 30 minutes. In contrast, 0.6% formalin required more than 72 hours to totally inactivate the neurotoxin.

Considerable progress has been made in determining the best conditions for the production of highly effective botulinal immunogens. In anticipation of the optimal formulation of these new toxoids, studies were initiated with the Aerobiology Division to test the efficacy of both the current and newly developed botulinal toxoids against an aerosol toxin challenge.

The initial evaluation in 23 volunteers of Botulinum Toxoid Adsorbed Monovalent (E), Lot #7007, produced by the Michigan Department of Public Health, was completed during this reporting period. Neutralizing activity for type E botulinum toxin was first detected in sera collected, from 11 of 12 volunteers receiving a complete immunization series, on day 91, which was 7 days after the third toxoid injection (TABLE I). The arithmetic mean titer was 0.298 IU/ml and by fourteen days after the third inoculation (day 98) the mean titer was 0.714 IU/ml. Ten of the eleven volunteers demonstrated substantial antibody responses after the third toxoid immunization; however, the eleventh volunteer did not seroconvert. Lot #7007 of the monovalent (E) botulinal toxoid proved to be a safe and efficacious product for the induction of substantial titers of neutralizing activity to type E botulinal toxin.

TABLE I. IMMUNE RESPONSES OF VOLUNTEER GROUPS INOCULATED WITH BOTULINUM TOXOID ADSORBED MONOVALENT (E), LOT #7007

VOLUNTEERS INOCULATED	DAY	ARITHMETIC MEAN TITER IU/ml[a]
11	0[b]	Undetectable
	14[b]	Undetectable
	28	Undetectable
	56	Undetectable
	84[b]	Undetectable
	91	0.298
	98	0.714

[a]International Units/ml serum. An *in vivo* (mouse) measure of serum neutralizing activity for type E botulinal toxin.
[b]Inoculation subcutaneously with monovalent (type E) botulinum toxoid, Lot #7007.

In a collaborative/contractural effort between LTC Lewis and MAJ Crumrine of USAMRIID and Dr. Richard Condie of the University of Minnesota the first two of four large production lots of Botulinum Immune Globulin (Human) Pentavalent (ABCDE) were fractionated. Lot #1A was derived from 226 liters of Botulism Immune Plasma (Human), Ind-1332. Lot #1A consists of 609.8 gm of IgG which has been divided among 530, 10 ml vials. This lot is $\leq$ 99% pure human IgG, non-pyrogenic, sterile, does not induce platelet aggregation, does not contain substantial anti-C^1 activity and is provided at 1.03 gm/vial, frozen. This product is labeled as Botulism Immune Globulin (Human) Pentavalent (ABCDE) and contains toxin neutralization activity for five botulinal neurotoxins. The product contains $\geq$ 99% monomeric IgG, which is a considerable improvement over commercially available human immune globulin products

such as Gamstan Human IgG, Bio-Test Intraglobulin I.V. Human Gammaglobulin, Cutter Modified Immune Globulin (Human) and the equine derived botulism antitoxin (ABE) produced by Connaught Laboratories.

Lot #1B has been fractionated and is currently being tested for neutralizing activity prior to bottling. Toxin neutralizing activity is expected to be equivalent to that of IVBG lot #1A.

A second collaborative/contractural effort with Dr. Condie involves a determination of the method(s) which will produce the best yields, highest toxin neutralizing activity, the most highly purified and the least immunogenic Heptavalent (ABCDEFG) Botulism Immune Globulin (Equine) product. To date, two pilot lots of heptavalent botulism immune plasma collected from the Army-owned horse "First Flight", have been fractionated by two different methods. Aliquots of the final immune globulin product were treated with different concentrations of pepsin for the purpose of determining which concentration of pepsin is optimal for preparing the least immunologically adverse (most completely despeciated) product having the highest neutralizing activity. Pepsin treatment reduces the intact IgG molecule to its $F(ab)^2$ fragment. However, these pepsin-generated fragments retain their ability to neutralize types A, B and E botulinal toxins, as is illustrated in Table II.

Five hundred liters of heptavalent equine plasma will be fractionated using the procedure employing 6% pepsin. High performance liquid chromatographic studies on pilot lots of this material show that the despeciation process results in significant conversion of the intact IgG to a 97,000 M.W. molecule. The resulting product contains few, if any, aggregates and few fragments.

The existence of antigenically distinct botulinal neurotoxins suggests probable differences in structure, biological activities and the structure-function relationship among these toxins. Two distinct "sites," one responsible for antigenicity and the other for toxicity, were detected for neurotoxins types A and E by a USAMRIID/MRDC extramural contractor, Dr. Don Das Gupta (7). Dr. Lawrence Sellin of USAMRIID then compared inhouse, on the basis of certain electrophysiologic parameters, the neurotoxicity of these two toxins. The study utilized highly purified neurotoxins (Mol. Wt. = 150,000), each produced by Dr. Das Gupta, and revealed considerable differences in the potency and duration of the effects of types A and E botulinal neurotoxins. Local blockade of transmitter release was produced by sublethal injection of either toxin when administered subcutaneously above the anterior tibialis muscle of adult male rats. At various intervals after injection, an extensor digitorum longus nerve-muscle preparation from a poisoned rat was examined for alterations in muscle mechanical properties (in vivo) or electrophysiological

TABLE II. BOTULINAL TOXIN NEUTRALIZING ACTIVITY IN TWO PILOT LOTS OF HEPTAVALENT BOTULISM IMMUNE GLOBULIN (EQUINE) TREATED WITH PEPSIN

LOT/SAMPLE	PROTEIN mg/ml	NEUTRALIZING ACTIVITY F $(ab)_2$[a] Toxin type A	B	E
Lot 07, 10 Nov 81				
Plasma	47.9	224	28	160
IgG 1% pepsin	21.3	282	56	127
IgG 3% pepsin	20.9	224	89	201
IgG 6% pepsin	19.8	282	89	201
IgG 9% pepsin	21.0	224	71	160
Lot 04, 15 Dec 81				
Plasma	17.1	224	48	144
IgG 3% pepsin	16.6	224	24	576
IgG 6%(a) pepsin	20.1	224	48	144
IgG 6%(b) pepsin	19.5	224	76	181
IgG 9% pepsin	50.2	224	48	181

[a]International Units as determined *in vivo* (mouse)

properties (*in vitro*). For both single twitch and tetanic tension, muscle treated with low (56 LD_{50}) doses of type E NT recovered from an initial depression of paralysis (induced with 565 LD_{50}) by 7 days after NT injection, while those treated with only 5 LD_{50} of type A remained significantly depressed through 10 days. Also, both miniature end-plate potential frequency and mean quantal content (m) were reduced to a greater extent and for a longer period of time for muscles treated with type A NT (3 LD_{50}) than for those treated with 20 to 282 LD_{50} of type E. Both type A and E botulinum NT produced a "denervation-like" syndrome. Type E neurotoxin was less effective in producing denervation-like effects than was type A neurotoxin.

This study demonstrated for the first time under the described experimental conditions, that purified botulinal neurotoxin type E is less potent, and in its effect is shorter in duration than type A botulinal neurotoxin.

A limited effort continues to address the development of chemotherapeutic approach to the treatment of botulism. Towards this end preliminary data generated during this reporting period suggest that the combined effect of 1 M 3,4-DAP and 10 uM neostigmine is more effective in antagonism of botulinal induced muscular paralysis than is 3,4 DAP or neostigmine alone.

During the past year substantial progress has been made toward the goal of developing a new and highly immunogenic anthrax vaccine. Numerous pilot lots of purified protective antigen (PA) have been prepared using a variety of new approaches. The purified PA has a molecular weight of ∿ 85,000 and a purity of 95%. This highly purified and soluble PA was converted to a more potent immunogen by adsorbing it onto an aluminum hydroxide gel adjuvant. Soluble and adsorbed PA preparations were evaluated as inducers of protective humoral antibody in the only standard model for such testing, the guinea pig.

A second major effort was undertaken in an attempt to develop a comparative data base on the efficacy of the American and British produced anthrax vaccines. This effort was collaborative in nature between British and USAMRIID scientists. Standard efficacy trials for the evaluation in guinea pigs of the two human anthrax vaccines vary considerably between the USA and the UK as to antigenic dose and immunization schedule. Results of the British evaluation are yet to be finalized. However, Dr. Anna Johnson-Winegar has made considerable progress on behalf of the USAMRIID effort, as can be deduced from Table III. The lot of American produced anthrax vaccine appears, in this initial pilot study, to induce higher titers to PA and to insure greater survival rates at all dilutions tested than does equivalent doses of the lot of British vaccine tested.

During this reporting period over 1,000 samples of human sera have been evaluated for the presence of anthrax (anti-PA) specific antibody. Table IV summarizes data from the titration of 190 of these sera, each collected at 14 days after completion of the initial series (3 immunizations) of immunizations with the American anthrax vaccine. The heterogenous humoral response is evidenced in Table IV and the data witnesses the need for a vastly improved anthrax immunogen in that 24% of those immunized responded with $\geq$ a 1:8 titer.

TABLE III. IMMUNE RESPONSE OF VACCINATED GUINEA PIGS

ANTHRAX VACCINE DOSE	TITER AFTER[a] VACCINATION	SURVIVORS/ TOTAL	TITER AFTER[a] CHALLENGE
American Vaccine			
1:2 dilution	512 (128-2048)	10/10	All 4096
1:6 dilution	1024 (128-4096)	11/12	All 4096
1:20 dilution	11.3 (Neg-64)	13/13	334 (16-4096)
1:60 dilution	64 (Neg-128)	14/14	37.6 (Neg-256)
British Vaccine			
1:2 dilution	25.4 (Neg-128)	13/13	19 (Neg-2048)
1.6 dilution	All negative	13/15	4.2 (Neg-512)
1:20 dilution	All negative	9/15	All negative
1:60 dilution	All negative	10/15	All negative
Saline	All negative	0/10	

[a]Geometric mean mean (range) at 14 days.

TABLE IV. IMMUNE RESPONSE OF VACCINATED HUMANS

N	TITER[a]
21	2
12	4
12	8
27	16
28	32
24	64
32	128
17	256
17	512

[a]IHA for PA, reciprocal of titer.

Considerable effort was devoted to the development of an ELISA for the rapid detection of humoral antibody (human) specific for the components of anthrax toxin. Initially, comparisons were made between the standard IHA and a first generation ELISA using our highly purified PA as the specific capture antigen. To date, pilot data generated only on the sera of immunized animals does not indicate a high degree of correlation between the two techniques. In part this lack of correlation may be explained by the apparent array of immunoglobulins induced by and specific for the heterogenous protein constitution of the standard anthrax vaccines. The IHA and ELISA techniques detect antibodies of different specificities. Preliminary experiments using the Western blot technique have revealed that sera from humans receiving anthrax vaccines contains antibody for a variety of extracellular proteins present in culture supernatants of B. anthracis.

Presentations:

1. Lewis, Jr., G. E. Passive acquisition of immunity to Clostridium botulinum type B toxin. Presented, Interagency Botulism Research Coordinating Committee, Philadelphia, PA, 5-6 October 1981.

2. Lewis, Jr., G. E. Evaluation of a botulinum toxoid (type B) in thoroughbred broodmares. Presented, 62nd Conference of Research Workers in Animal Diseases, Chicago, IL, 7-10 November1981.

3. Lewis, Jr., G. E., S. S. Kulinski, D. W. Reichard and J. F. Metzger. Detection of Clostridium botulinum type G toxin by enzyme-linked immunosorbant assay. Presented, American Association of Equine Practitioners, New Orleans, LA, December 1981.

4. Johnson-Winegar, A. D. Evaluation of the protective antigen component in establishing immunity to anthrax. Presented, Review of the USAMRIID Anthrax Program, WRAIR, Washington, DC, 1 December 1981.

5. Johnson-Winegar, A. D. Immunizing activity of the protective antigen component of Bacillus anthracis. Presented, Professional Staff Meeting, USAMRIID, FT Dettick, MD, 28 January 1982.

6. Sellin, L. C. The action of botulinum toxin at the neuromuscular junction. Presented, Biology Department, Seton Hall University, South Orange, NJ, 14 October 1981.

Publications:

1. Lewis, G. E., Jr. 1981. Editor, Biomedical Aspects of Botulism. Academic Press, Inc., NY.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL DD-DR&E(AR) 634
				DAOG 3813	82 10 01	
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
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10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	BB	149
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE *(Precede with Security Classification Code)*
(U) Prevention of Bacterial and Rickettsial Diseases of Potential BW Importance

12. SUBJECT AREAS
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	10.2	568
c. TYPE	d. AMOUNT	83	17.0	1208
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Bacteriology Division, USAMRIID
b. ADDRESS *(Include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR Hedlund, K W
d. TELEPHONE NUMBER *(Include area code)* 301663-2833	d. TELEPHONE NUMBER *(Include area code)* 301-663-7341
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Rozmiarek, H
MILITARY/CIVILIAN APPLICATION:	g. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Jemski, J V

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Bacterial Diseases; (U) Rickettsial Diseases; (U) Vaccines; (U) Laboratory Animals

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Develop a base of technology and when possible, specific measures that either prevent or effectively counteract bacterial and rickettsial diseases that fall into 2 categories; have significant BW potential and could be used against U.S. Forces because of their vulnerability; and are highly virulent and dangerous agents which require special containment laboratories for safe study and are uniquely important to the U.S. military. To produce and purchase experimental animals of high quality to implement these studies.
24. (U) Program objectives are multifaceted: to develop safe and effective vaccines for those bacteria and rickettsiae which have BW potential; to conduct studies on respiratory immunizations; to study immunity derived by sequential immunizations of two or more vaccines; to develop appropriate adjuvants for use with weakly antigenic vaccines; to exploit any emerging technology that is concerned with modifying the immune system to the advantage of the host.
25. (U) 8110 - 8209 - The isolation of N-acetylglucosamine galactose polysaccharide from *B. anthracis* seems to be unique to to this species. This constitutes an important discovery because the presence of this sugar can be used to quickly identify anthrax spores from other closely related but non pathogenic spore formers. Investigations are under way to isolate and characterize the immunogenic components of *Coxiella burnetii* which should provide the knowledge to produce an improved vaccine for Q fever. The *Legionella pneumophila* program has met program objectives, is 90% complete and is being phased-out. The animal disease surveillance program functioned normally during the year and quality animals are being provided for the Institute's research programs. A variety of experimental anthrax vaccines are being evaluated and compared for ability to protect laboratory animals against different types of exposure to virulent anthrax bacilli.

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)

Work Unit No. 871 BB 149: Prevention of Bacterial and Rickettsial Diseases of Potential BW Importance

Background:

This annual report is the summary of four individual's annual reports. Since they have their own particular areas of emphasis each research unit will be treated individually. Because of a similarity of subject matter an addendum about ongoing anthrax projects will be included here prior to its acceptance as a formal research unit.

COL Rozmiarek has two research work units, the first deals with hazards and variables associated with research animals, a problem for an infectious disease research institute, because contamination may be masked by the effects of the conditions under study, and may, in turn, change the results of the research study. This potential for contamination is amplified at USAMRIID where space limitations generally preclude in-house breeding programs, and all conventional animals are purchased from commercial sources whenever available.

During this period pasteurellosis was discovered in rabbits; pinworms in rabbits, mice and hamsters; coccidiosis in rabbits; hexamitiasis in mice and hamsters; and encephalitozoonosis in rabbits.

With the many varied and unusual organisms used at USAMRIID, occasional diseases or organisms are encountered for which no animal model or natural reservoir is known. In some instances a model is known to exist, but no reliable commercial source for that particular animal species exists. If no animal is known, investigations must be carried out to determine what species would be acceptable. If an available model is a problem, in-house colonies must be established to meet investigators needs.

Several rodent species not available commercially have been identified as models for diseases under investigation at the Institute. The Calomys callosus (vesper mouse) is the natural reservoir host for many arenaviruses, including Machupo virus, and is the only known species colonized which allows Korean hemorrhagic fever virus to grow and replicate. Sigmodon hispidus (cotton rat), is a natural reservoir for VEE and Tamiami viruses.

The gappers red backed vole, Clethrionomys gapperi, has been identified as a potentially valuable animal model to be used in the study of several viruses currently being investigated by the Institute. This animal is native to arctic North America and several of the Scandinavian countries, but all efforts to date to raise it in captivity have been unsuccessful.

Inbred Strain 13 guinea pigs (Cavia porcellus) have been identified as suitable animal models for several viruses being investigated at USAMRIID, including Junin virus, Machupo virus, Lassa fever virus, Ebola virus, and Pichinde virus. An in-house production colony has been maintained for several years to meet investigator demands because sufficient numbers of Strain 13 guinea pigs cannot be obtained

commercially. Reproduction in this guinea pig strain is relatively poor compared to outbred guinea pig stocks and therefore requires intensive husbandry and management for successful production. Expanded use of the Strain 13 guinea pig as a suitable animal model for Pichinde virus has increased the production requirements.

Dr. James Johnson is terminating this work unit on the characterization of a Q fever vaccine. These Q fever vaccines characteristically have been purified or partially purified preparations of phase II or phase I rickettsiae obtained from infected yolk sacs of embryonated hens eggs. The vaccine in current use is a phase II preparation that was developed about 20 years ago (1, 2). This vaccine provided good protection against the disease, but caused a significiant number of systemic or local reactions among recovered or previously immunized individuals. Supplies of this vaccine have diminished, and a replacement will soon be needed.

Since this work unit is being terminated, a final report is being written summarizing the information developed and the progress made.

Physical properties. Direct rickettsial counts of 5 lots of phase I and 1 lot of phase II vaccines were determined. Concentrations of organisms ranged from 3.2 to 3.6 X 10^9 organisms/ml for the phase I lots and was 1.5 X 10^9 for the phase II vaccine. Buoyant densities, in CsCl, of the phase I vaccine lots were all 1.33 gm/ml with a single band being observed in each gradient. The phase II vaccine had a diffuse band between density levels 1.19 and 1.25 gms/ml and a sharper, less dense band at 1.33. The optical density of the phase I vaccine lost varied from 0.22 to 0.27 OD units, and was 0.42 for the phase II vaccine.

Dose. The median serologic doses, the g of vaccine needed to elicit a minimum significant antibody response in one-half the guinea pigs by complement fixation (CF), microagglutination (MA), or immunofluorescent antibody (IFA) procedures, were determined. Phase I vaccinated guinea pigs required 19 µg for a median MA dose, but 30 µg for CF and IFA doses, using a phase I antigen. With a phase II antigen, median doses were > 30, 7.3, and 1.4 µg for CF, MA, and IFA tests, respectively. For phase II vaccinated guinea pigs, median doses > 30 µg were needed with either phase I or phase II antigens.

Reactogenicity. Comparative skin test studies in guinea pigs of 6 lots of the phase I and the phase II vaccine were conducted. Intradermal injection of various amounts of vaccine in Q fever sensitized guinea pigs caused a progressive development of induration at the inoculation site. The degree of induration was quantitated by measuring skin thickness at the injection site and following its development over several weeks. Lot 4 of the phase I and the phase II vaccines were compared and found to give similar minimal but detectable skin reactions with 0.1 µg of vaccine, the smallest amount used. The 5 lots of the phase I vaccine were also compared, and all gave easily measured skin reactions with 0.3 µg of vaccine.

Median protective dose. Estimates of median protective (PD_{50}) doses of the phase I vaccine lots ranged between 1.8 and 0.3 µg of vaccine. The phase II vaccine was estimated to require about 27 µg of vaccine for a PD_{50}. These values are only approximations because of errors in the assay due to nonspecific fever responses observed in control guinea pigs. A number of guinea pig sources, changes in housing and environmental conditions of the animals and other parameters of infection such as variations in the concentrations of serum enzymes during infection have been evaluated in an attempt to improve the reliability of the protective dose estimates.

Dr. Ezzell is working on the immunogenicity of Bacillus anthracis. The present human vaccine against anthrax consists of an alum percipitate of the culture supernatant of Vollum 770 strain B. anthracis. The primary constituent of this vaccine is the protective antigen component of the tripartite toxin of this organism. However, it is generally agreed that live attenuated vaccines used by veterinarians for vaccination of livestock provide better protection (3). This led to the speculation that antigens in addition to the protective antigen component of the toxin may be efficacious as immunogens against anthrax. Initially, purified cell wall material is being tested which chemical data suggest to contain a major galactose containing polysaccharide described by Ivanovics (4) and Smith, et al. (5). Also described within a e studies on rapid identification of B. anthracis, effects of anthrax bacterial products on phagocytic functions during disease, chemical analyses of anthrax cell walls and biological sterilization of soil contaminated with B. anthracis spores.

The goal of our overall anthrax research effort is to maximize protection against anthrax by means of improved immunization and detection. Thus, work done by Dr. Ivins, MAJ Ristroph, and Captains Mikesell and Drier will be noted in the progress section pending approval of a formal work unit.

Progress:

The animal disease surveillance program under COL Rozmiarek's research functioned normally during this period, with the quality control monitoring of 516 rats, voles, gerbils, mice, hamsters, guinea pigs and rabbits from 12 different commercial sources as well as 3 USAMRIID breeding colonies. This total represents 45 strains and stocks from the above number of commercial sources. All animals were examined for gross and histologic pathology, bacterial pathogens, parasites and hematologic parameters. The hematological data is used to establish normal values for each strain/stock of rodent used at USAMRIID and to determine abnormal values which may indicate latent metabolic or infectious disease processes.

The following diagnostic specimens (Table I) were examined during this period:

TABLE I. DIAGNOSTIC SPECIMENS

TYPE EXAMINATION	NUMBER
Hematology	1,447
Parasitology	993
Bacteriology	782
Histopathology	516
Serology	50
Total	3,788

The most frequent clinical conditions detected in newly received animals this period were in rabbits. Several animals were rejected because of chronic respiratory disease (CRD) caused by Pasturella multocidea. This disease poses a serious threat to those rabbits already housed in USAMRIID because it is difficult to cure the clinically ill, and equally as difficult to eradicate once the disease has established itself in the colony. The incidence of ear mite infestation was low during this period although several recurring infections were treated.

Encephalitozoonosis (Encephalitozoan cuniculi) was diagnosed in 3 rabbits during this reporting period including one rabbit exhibiting uncoordination and spastic paralysis. There is no treatment for infection. The infected animals must be eliminated from the colony. No further incidence has been noted.

Several spontaneous deaths occurred in the rabbit colony: acute peritonitis secondary to gastric trichobezoar (hairball); acute pneumonia (3 cases) due to Pasteurella multocida or Bordetella bronchiseptica infections; and traumatic vertebral fractures (3 cases). Respiratory disease and chronic conjunctivitis caused by Pasteurella multocida continues to be prevalent. The continued use of autoclaved hay has seemingly reduced the incidence of "anorexia/lack of stool syndrome" associated with hairballs. The feasibility of feeding alfalfa cubes to replace the autoclaved hay has been tested and appears to be just as effective in preventing hairballs.

Several shipments of Swiss mice and one shipment of hamsters were found to be infected with hexamitiasis (spironuceosis) caused by the protozoan Hexamita muris. The supplier was not aware of the infection in the colony. The problem will be corrected in future orders. No significant pathological changes were reported in these infections.

An outbreak of Bordetellosis (Bordetella bronchiseptica) was diagnosed in a group of newly-arrived guinea pigs. The onset of clinical disease was attributed to shipping stress. Subsequent orders of guinea pigs from this supplier were made only from specific pathogen-free, barrier-reared (BR) colonies rather than open colonies. In addition, all future orders were shipped in protective filter-lined shipping boxes. These measures, along with strict sanitation and disinfection procedures, have resulted in no new outbreaks.

An adenovirus was identified on electron microscopy from the lungs of two Hartley guinea pigs [Crl: (HA)BR] that died of acute pneumonia. This was extremely significant since there has been only one previous report of adenovirus-induced respiratory disease in guinea pigs and none reported in the U. S. Three other guinea pigs in the group of 118 also died but were not examined histopathologically. No other outbreaks of respiratory disease have been observed in guinea pigs from this supplier. However, the virus may have originated within USAMRIID or during transit. All unexpected spontaneous deaths of guinea pigs are being necropsied as usual to ensure that this was only an isolated outbreak.

Data concerning the hematologic characterizations of naturally occurring malarial (Plasmodium inui) infections in cynomolgus monkeys was compiled and analyzed. All infections were subclinical in nature. Parasitemias ranged from 10-900 parasites/nm^3 of whole blood. Pre- and post-treatment hematologic values were evaluated following treatment with chloroquine. Treatment was effective in clearing

13 of 14 infected monkeys. Pre-treatment values of hematocrit, hemoglobin and mean corpuscular volume were significantly different in infected monkeys compared to noninfected monkeys. While post-treatment hemoglobin and hematocrit values returned to noninfected control ranges, mean corpuscular volume values of infected monkeys remained significantly lower in the post-treatment period.

Data collection was completed for a study designed to determine the effects of naturally-occurring chronic malaria on the immune system of cynomolgus monkeys. These infections have been shown to persist for over five years after importation and have been found in up to 43 percent of cynomolgus monkeys imported for use of USAMRIID. The effects of this infection on biomedical research at USAMRIID are unknown; however, data from humans with chronic malaria and experimentally infected rhesus monkeys (Macaca mulatta) suggests that malaria causes immunosuppression and decreased antibody response to certain vaccines. Data has now been collected from eight infected and eight noninfected control monkeys before and after treatment with chloroquine. Parameters examined included T and B lymphocyte stimulation indexes. This data is being compiled for statistical analysis.

A collaborative study with Dr. Peter Jahrling in Virology Division was performed to determine if cynomolgus monkeys (Macaca fascicularis) with chronic malaria had a different response to Lassa fever virus infection compared to noninfected controls. No significant differences in onset and severity of viremia, antibody response and time to death were observed. An unexpected observation was a decrease in parasitemia following virus challenge and subsequent undetectable counts in three of four monkeys. It was originally thought that the malaria would have increased the severity of the Lassa fever infection or vice versa, but this was not evident. Pigment found in tissues on histopathology was able to be definitively attributed to the malaria because the malaria status of these monkeys had been determined prior to the experiment. Investigators using potentially-infected nonhuman primates should be aware of possible complicating effects such as this.

A bifunctional typhoid fever and bacillary dysentery oral vaccine developed by the Walter Reed Army Institute of Research was safety tested in rhesus (Macaca mulatta) monkeys. This live vaccine consisted of a nonpathogenic mutant Salmonella typhi strain transconjugated with a protective antigen from Shigella sonnei. The vaccine study demonstrated that the vaccine did not result in diarrhea or other clinical illness. Since no adverse reactions were observed, human volunteer studies are being planned. This vaccine should give protection against both typhoid fever as well as dysentery caused by S. sonnei.

Under COL Rozmiarek's research, the primary functions of the Sigmodon hispidus and Callomys callosus rodent colonies were to meet the documented demands of the Institute for these commercially nonavailable animals for investigative needs. We have achieved this goal and are continuing to provide vesper mice and cotton rats to using investigators. No major problems associated with disease, husbandry, or reproduction were encountered during the reporting period. See Table II.

TABLE II. STATUS OF SPECIFIC RODENT COLONIES

	Sigmodon hispidus (about 25 breeding pairs maintained)	Callomys callosus (about 25 breeding pairs)
Born	180	285
Weaned	153	259
Issued	105	78
Sacrificed	1	205

A colony of Grappers red backed voles, Clethrionomys gapperi, has been established at USAMRIID to support hemorrhagic fever virus studies. During this period there were 255 animals born, 210 animals weaned and 246 animals issued to the Virology Division. See Table III.

TABLE III. STATUS OF VOLE COLONY

	Clethrionomys gapperi
Born	255
Weaned	210
Issued	246
Sacrificed	20

The breeding colony of strain 13 guinea pigs (Cavia porcellus) has become stabilized over the last year to 30 breeding racks or 150 harems usually consisting of four females and one male per harem. There were 548 breeding females at the end of the fiscal year. The colony is presently located in six animal rooms in Suite AR 3 in Bldg 1425. There were 2,155 offspring born this past year with 584 of these being retired breeding stock from NIH and the USAMRIID colony. As in the past, we are maintaining genetic uniformity by using exbreeders from the inbred colony maintained in the Division of Research Services at NIH. These breeders are randomly mated and offspring retained for up to only four generations.

In late 1980, there was an epizootic of pneumonia caused by Bordetella bronchiseptica which caused significant morbidity and some mortality. This outbreak was brought under control with an autogenous bacterin and there have been no further outbreaks. Recently, Klebsiella cobayae has been observed in histological sections of kidneys from Strain 13 guinea pigs which were in various research protocols. This organism is a coccidian, which is common in the kidney and other organs of guinea pigs and is considered to be nonpathogenic. We are currently determining how widespread this infection is in our colony and if indeed it is nonpathogenic.

Guinea pig cytomegalovirus (salivary gland) virus infection has been detected on histopathology. Although this infection causes no clinical signs, it can interfere with ultrastructural observations concerned with determination of the pathogenesis of other diseases such as the arenaviruses. The common fur mite of guinea pigs, Chirodiscoides caviae, has been identified in both guinea pigs from NIH as well as the USAMRIID colony. This mite is not thought to be pathogenic and successful elimination is only by cesarean rederivation. Enzootic guinea pig inclusion conjunctivitis (Chlamydia psittaci) continues to persist in the colony. This disease affects young guinea pigs less than 3 weeks of age. Severe cases are treated with chloramphenicol ophthalmic ointment and resolve within a few days.

Negotiations are currently under way with the National Cancer Institute of NIH to establish a cesarean-derived, barrier-maintained, strain 13 guinea pig production colony at Fort Detrick. This colony would eventually supply all of the strain 13 guinea pigs for USAMRIID. Successful germ-free derivation of this strain would eliminate the above-mentioned parasitic, cytomegalovirus, and chlamydial infections and result in much healthier research animals free of extraneous confusing lesions. See Table IV.

TABLE IV. STRAIN 13 GUINEA PIGS ISSUED DURING FY 82

INVESTIGATOR	DIVISION		
	VIROLOGY	BACTERIOLOGY	MEDICAL
LTC Lupton	191		
Dr. Kenyon	267		
Dr. Liu	156		
Dr. Jahrling	300		
Dr. E. Johnson	176		
LTC Peters	30		
CPT Huggins	40		
Mr. Pannier	30		
Dr. Ezzell		100	
LTC Cosgriff			32
	1,190	100	32
Total Issued:	1,322		

Primate colonies. The rhesus breeding colony has continued to produce healthy rhesus offspring in addition to providing valuable experience in the management and maintenance of a primate breeding colony. Growth, maturation and hematologic data continue to be collected on first and second generation progeny.

There were no deaths recorded during the reporting period. There was one live birth, on 4 February 1982. Breeder #0732 delivered a healthy female baby (#B66). She is currently nursing the baby. There are two females pregnant.

The colony is being maintained at 2 breeding males and nine breeding females. At present, there is one nursing infant, ten juvenile females, ten adolescent females (one menstruating), and 29 juvenile males. No significant health or husbandry problem was noted during the reporting period. The colony reared females which have begun to cycle are within normal limits on the basis of physiological parameters. Observations of psychological behavior will continue on our colony raised offspring.

Dr. James Johnson's research ended with an evaluation of the physical characteristics of an experimental Q-fever vaccine. Direct rickettsial counts of the vaccine lots were conducted according to the procedure of Silverman et al (6). Vials of vaccine were reconstituted with saline. A suspension of killed Shigella dysentereae containing 3.1×10^9 organisms/ml was used as the standard. Organisms in droplets were counted to yield a minimum of 500 rickettsiae. From these data and the known Shigella concentration, the rickettsial concentrations were calculated. See Table V.

Buoyant densities were obtained with CsCl gradients according to the procedure of Wachter et al (7). One ml of reconstituted vaccine was layered on the surface of 11 ml of CsCl solution (1.33 g/ml) and tubes were centrifuged at 105, 000 X g for 24-36 hrs in a Beckman type 41 rotor. Each lot of the phase I organisms were expected to band. No other bands were observed. By contrast, the phase II vaccine showed 2 bands: one being rather dense but diffuse between densities of 1:19 and 1.25, and the other being less dense and sharper at 1.33 gm/ml. The phase II vaccine was prepared originally using a predominately phase II strain of C. burnetii; therefore, the diffuse band at this density range was expected, since this is characteristic of phase II organisms. The band present at the 1.33 gm/ml density suggests either a phase I component in the vaccine or more likely, the presence of phase II organisms from which the buoyant host material had been removed during the purification or gradient procedures. Buoyant densitites of the vaccines are shown in Table V.

Optical density of the vaccines were measured at 420 m using a Beckman DB spectrophotometer and a path length of 10 mm. This is the wavelength and instrument used by Fiset et al. (8) for standardizing rickettsial suspensions for microagglutinations tests. All 5 lots of the phase I vaccine had OD between 0.25 and 0.27 when reconstituted. These values were used to calculate the number of organisms/ml and the number of µg/ml in the vaccines. Fiset et al. (8) reported that 100 µg/ml of purified C. burnetti had an OD of 0.33 using conditions similar to those above. Using these numbers as a ratio to calculate the number of µg/ml of rickettsiae in the vaccine lots, values close to 80 µg/ml were obtained for the phase I vaccine lots. This suggests that either there is host material left in the vaccine or that the values reported by Smith, et al. (5) were too low (Table I).

TABLE V. PHYSICAL PROPERTIES OF Q FEVER VACCINES

VACCINE	BUOYANT DENSITY[a] (gm/ml)	OPTICAL DENSITY[b]	RICKETTSIAE/ML DRC x 10^9	RICKETTSIAE/ML OD[c] x 10^9	G/ML BY OD[d]
Phase I					
Lot 1	1.33	0.26	3.5	3.7	78
Lot 2	1.33	0.27	3.7	3.9	82
Lot 3	1.33	0.25	3.3	3.6	76
Lot 4	1.33	0.27	3.2	3.9	82
Lot 5	1.33	0.27	3.6	3.9	82
Phase II					
DP-7	1.19-1.25 1.33	0.42	1.5		

[a] In CsCl.
[b] Beckman DB-G spectrophotometer, 420 nm, 1 cm path length.
[c] Calculated from assumption that 4.7 X 10^9 rickettsiae/ml = 0.33 OD units, Fiset (8).
[d] Calculated from assumption that 100 µg rickettsiae/ml = 0.33 OD units, Fiset (8).

By contast, Heggers, et al. (9) reported that there were 4.7 X 10^{10} C. burnetii/ mg of dry weight. Therefore, 100 µg of vaccine should contain about 4.7 X 10^9 organisms, and should exhibit an OD of 0.33. If these values are used to calculate the number of organisms/ml, estimates of 3.6 X 10^9 and 3.9 X 10^9 C. burnetii/ml are obtained. These values agreed fairly well with the direct rickettsial count as shown in Table I.

Some immunological properties of these vaccines were investigated in guinea pigs to estimate effective doses needed for antibody production and to determine whether humoral or cellular immune responses could be related to susceptibility to infection. The complement fixation (CF, 10), microagglutination (MA, 8) and immunofluorescent antibody (IFA, 11) tests were used for humoral immunity studies and the lymphocyte transformation (12) and skin reactivity tests for cell mediated immunity. In initial studies, the phase II and lot 4 of the phase I vaccine were compared for their ability to elicit antibody responses in guinea pigs. Vaccine doses needed to induce at least a minimum antibody response in one-half the guinea pigs were determined. Groups of 10 guinea pigs were vaccinated i.d. with 0.5 ml of selected doses of each vaccine. On the 20th day after injection the guinea pigs were bled by cardiac puncture and CV, MA, and IFA tests were determined for the sera. Median serologic doses were calculated by the Reed-Muench method using the number of guinea pigs in each group having titers of 1:10 or greater. The results of these tests are shown on Table II.

The phase II vaccine required > 30 µg, the largest dose tested, to produce an antibody response in any of the tests using either phase I or phase II antigen. Phase I vaccine produced a detectable response to phase I antigen only in the MA test. With the phase II antigen, measurable MA and FA antibody responses were obtained, but the CF response remained > 30 µg.

Microagglutination tests were conducted on the sera of guinea pigs receiving various doses of the 5 lots of the phase I vaccine. Groups of 8 vaccinated animals were bled on day 20 postvaccination and their sera tested for phase I and phase II Q fever antibodies. See Table VI.

TABLE VI. MEDIAN SEROLOGIC DOSES OF PHASE I AND PHASE II Q FEVER VACCINES IN GUINEA PIGS

ANTIGEN TEST	MEDIAN DOSE - G OF VACCINE VACCINE PHASE	
	PHASE I	PHASE II
Phase I		
Complement fixation	30	30
Microagglutination	19	30
Immunofluorescent anitbody	30	30
Phase II		
Complement fixation	30	30
Microagglutination	7.3	30
Immunofluorescent antibody	1.4	30

The data presented are the geometric means of the reciprocal MA titers of each group of guinea pigs. Antibody titers generally increased with increasing doses for both Q fever phases, although changes were minimal at the lower doses. For detectable phase I antibody to be produced, between 0.35 and 1.7 µg of antigen were required under the conditions of the experiments. Phase II antibodies were produced by much lower doses, in the order of 0.0001 µg of vaccine.

A study was conducted to estimate the vaccine dose needed to cause the development of a skin reaction in guinea pigs following the i.d. injection of 30 µg of antigen. Groups of normal guinea pigs were vaccinated i.e. with a single dose of either 30, 3.0, or 0.3 µg of phase I, lot 5, vaccine. Six weeks later the guinea pigs were challenged i.d. with 0.1 ml containing 30 µg of phase I, lot 5, vaccine. Skin thickness was measured at each injection site at various time intervals following injection. The results of the experiment is shown in Table VII. These data indicate that a phase I vaccine dose of 0.3 µg per guinea pig can sensitize the animal to a later injection of antigen. Increasing the sensitizing dose causes the reaction to become more severe, to begin at an earlier time and to last longer. The control guinea pigs which received the 30 µg challenge dose but no sensitizing

vaccination did not show observable skin reactivity until about 2 weeks. A slight increase in skin thickness was noted which increased somewhat at later times. This could be due to some C. burnetii remaining fixed in the skin lesions as the animal begins to develop immunity to the vaccine antigen.

TABLE VII. DOSE OF VACCINE NEEDED FOR THE DEVELOPMENT OF A SKIN REACTION IN GUINEA PIGS CHALLENGED I. D. WITH 30 μG OF ANTIGEN

	MEAN SKIN THICKNESS MM VACCINE DOSE[4] μG			
DAY	30 n = 8	3 n = 12	0.3 n = 8	0 n = 8
1	0.48	0.25	0.44	0.18
2	0.36	0.32	0.24	0.09
3	0.53	0.35	0.22	0.13
7	1.12	0.64	0.10	0.19
10	1.50	1.02	0.23	0.15
14	1.95	1.34	0.89	0.63
17	1.78	1.63	1.30	0.33
21	2.08	1.69	1.32	0.55
25	2.13	2.14	1.09	0.35
29	1.97	1.90	1.09	0.40
32	1.63	1.90	0.99	0.33

Initial experiments comparing the protective properties of the phase II and phase I Q fever vaccines in guinea pigs indicated that a number of problems needed solving before adequate precision in the comparisons could be made. One problem was the occurrence of brief sporadic increases in the body temperature of guinea pigs before vaccination or challenge. Since temperature after challenge was the criterion used for determining infection, these sporadic changes increased the error of the experiment. It was found that an acclimatization period of at least 2 weeks was needed, during which time the animals were kept in individual cages, handled frequently, and allowed to become adjusted to the new surroundings. The optimal temperature of the holding rooms was determined to be 70°F (21°C) because higher temperatures caused occasional temperature spikes and lower temperatures tended to increase the incidence of respiratory infections with associated febrile responses. Also, the source of guinea pigs was important. In this study, 3 sources were tested, West Jersey Biological Co., Buckburg, Inc., and Charles River, Inc. The West Jersey animals were in poor condition on arrival, most had or soon developed, respiratory infections and several died. Buckburg guinea pigs arrived in

good condition and remained so throughout the holding period, except for an occasional temperature spike. Charles River guinea pigs were in excellent health on arrival and remained so throughout the holding period. These latter animals were used in the following studies.

Because febrile reactions in guinea pigs are so sensitive to a variety of sometimes uncontrollable influences, biochemical changes in serum were investigated as supplements to or alternatives for this indicator of infection. Heggers, et al. (9) reported that Q fever infection in the guinea pig affected a number of serum components. Glucose, glutamic-oxalacetic transaminase (GOT), and creatine phosphohinase (CPK) values appeared to be most changed by the infection and were chosen for further study along with lactic dehydrogenase (LDH), and creatinine. Most of these measures are associated with liver or cardiac functions, the primary organs involved in Q fever infection. Experiments were designed to study changes of these biochemical parameters throughout the course of illness in Q fever infected guinea pigs. Three groups were used, a control group with no infection, a low dose group receiving 10^2 ID_{50} of C. burnetii and a high dose group receiving 10^5 ID_{50}. Temperatures were recorded daily for all animals and selected guinea pigs from each group were bled daily for serum. Serum glucose concentrations increased noticeably in the high dose group by day 3 post infection and seemed to parallel the febrile response. In the low dose group, glucose values were slightly elevated but showed less correspondence with fever. Serum GOT activity in the high dose group was elevated between days 8 and 11 post infection, which was several days after the peak fever response. The low dose group had no significant elevation of serum GOT activity. Between days 5 and 9 post infection, CPK activity in the high dose group was somewhat depressed compared to the control group, while the activities of the low dose group paralleled the controls fairly closely. LDH and creatinine values in both infected groups and the control group were similar.

Of the biochemical parameters investigated, only glucose concentrations appeared sufficiently altered to be useful in indicating Q fever infection severity in the guinea pig. The sensitivity of the change to mild infections, which may be encountered in partially protected animals, was not very high. The values of other serum components were either too variable or required too severe an infection to show significant changes. However, a group of properly selected serum components, some of the above and perhaps other, along with temperature changes, may provide a reasonably reliable but indirect means for quantitating Q fever infection in the guinea pig if a lethal model cannot be developed in a reasonable time.

Dr. John W. Ezzell has a multifaceted approach to problems of immunogenicity of Bacillus anthracis. Each area of research endeavor is summarized as follows:

Vaccine development. Antigens other than the protective antigen component of the toxin may be efficacious as immunogens against anthrax. Initial studies indicated that purified cell wall from B. anthracis, Sterne strain, provides up to 80% protection in guinea pigs against parenteral challenge (IM) with virulent Vollum 1B strain spores. The mode by which the vaccine is administered is important if one is to obtain an adequate titer for protection. A micro-agglutination assay for determining anti-cell wall titers was developed which is proving invaluable for such studies. Also, Lewis rats are being administered cell wall material to determine if this antigen is arthritogenic. Thus far, the cell wall material has not produced arthritis after 5 weeks.

Chemical composition studies of Bacillus species cell walls. Chemical analysis of purified cell walls have revealed that B. anthracis differs from the two closely related species, B. cereus and B. thuringiensis. B. anthracis contains high levels of galactose (20-25% w/w) whereas the other two species contain no galactose but instead contain glucose (3-5% w/w) and trace levels of galactosamine. Collaborative studies with Dr. R. J. Doyle, University of Louisville, Louisville, KY, have shown the glucosamine in B. anthracis peptidoglycan is not acetylated which is required for the action of lysozyme. This explains the chemical basis of resistance by B. anthracis to lysozyme which is used in selective media, e.g., PLET medium (4) for this organism.

Macrophage and polymorphonuclear neutrophil studies. Gamma irradiated B. anthracis vegetative cells (capsulated and non-capsulated) and spores plus various antisera are being supplied by this investigator to the following collaborative studies. In studies with MAJ John O'Brien, Physical Science Division, USAMRIID, the chemiluminescent response to human PMNs upon exposure to virulent and avirulent cells of B. anthracis strains opsonized with antisera directed to various antigens (i.e., capsule, cell wall, protective antigen, etc.) is under investigation. LTC Arthur Friedlander, Bacteriology Division, USAMRIID, is studying the effect of capsule and toxin on the activity of macrophage growth factor and fusion in macrophage of lysosomes with phagosomes which contain ingested B. anthracis cells. Finally, Mr. Werner Janssen is studying the effect of toxin and capsule on phagocytosis of B. anthracis by various phagocytic cells.

Rapid identification of B. anthracis. Studies demonstrated that fluorescein conjugated antisera to B. anthracis Sterne strain cell wall selectively strains B. anthracis. Low level cross-reactivity with other Bacillus species is removed by adsorption with B. cereus ATCC 6464 cell wall. Although most B. cereus and B. thuringiensis strains do not stain, a few strains have been found that strongly cross-react and areindistinguishable from B. anthracis when identification is based soley on this FA. These strains include B. cereus ATCC 23260, ATCC 19637, ATCC 7064, NRS-820 and B. thuringiensis ATCC 4045. B. cereus NRS-820 was found to be a mislabeled B. anthracis strain in that it produces B. anthracis toxin components, has a typical cell composition for anthrax and resembles B. anthracis in many other respects. However, it does not produce a capsule. The other cross-reacting strains are motile, hemolytic, do not form capsules and have a cell wall chemical composition like other B. cereus and B. thuringiensis strains. In addition, these abberant strains do not produce the protective antigen component of anthrax toxin as determined by a double agar diffusion immunossay developed by Dr. Bruce Ivins. In this technique, 1 mm wells are cut 5 mm from a 48 h colony growing on R medium agar into which goat antisera to the Michigan antigen (protective antigen) is added. The precipitin lines which formed did not produce discernible lines of identity with authentic protective antigen. The nature of the cross-reacting surface antigen of these abberant strains is not known since all chemical data to date indicates that they resemble other members of their respective species.

Cell wall antisera is presently being produced in goats, because it is believed that rabbits do not respond to the galactose containing polysaccharide of B. anthracis which chemical data indicate may be a major cell wall component. If goats respond to this polysaccharide, as has been demonstrated in horses, the cross-reactivity problems may be circumvented because FA against this polysaccharide should stain only B. anthracis. Monoclones are also being developed in collaboration with

Biotec Laboratories, Rockville, MD, against B. anthracis Sterne strain cell wall as a second possible means of circumventing cross-reactivity problems. Currently, a dual system is being used to identify virulent capsulated B. anthracis based on the staining of capsule around cells grown on nutrient agar containing 0.8% sodium bicarbonate and staining of the cell wall of cells grown on regular nutrient agar.

Chemical studies by this research unit, which demonstrated B. anthracis cell wall to contain high levels of galactose suggested that galactose specific lectins may be useful in identification of this organism. Collaborative studies with Dr. Doyle have demonstrated that B. anthracis can be differentiated from these closely related species via galactose specific lectins.

Additional work on anthrax involves the research efforts of Dr. Bruce Ivins, MAJ Joseph D. Ristroph, Captains Perry Mikesell and Thomas Drier as well as Mr. Werner Janssen. The individual efforts will be covered by topics.

Development of a new synthetic medium. A new synthetic medium composed of amino acids, salt, glucose, purines, and pyrimidines was developed (Table VIII). The parameters of growth and toxin production have been monitored in two vaccine strains and a virulent strain. The production of the holotoxin and its LF and PA components were monitored, using the double-diffusion Ouchterlony technique and the rat lethality assay, in two commonly used media as well as the new medium (R medium, Table IX).

Plasmid isolation and transformation. Because many bacteria use extrachromosomal DNA for toxin regulation, several strains of B. anthracis were examined for this type of DNA. Two encapsulated, toxigenic strains B. anthracis (Weybridge and V-770-NP1-R) and a virulent strain (Vollum 1B) were cultured and passaged for ten consecutive days at 42.5°C in R medium. At each passage, a 0.05-ml culture aliquot was inculated into 5.0 ml of fresh media and incubated further for 24 h. Following the tenth passage, individual colonies from these broth cultures were isolated on sheep blood agar, then inoculated into 125 ml of fresh broth media (10^3 colony-forming units per ml), and incubated for 18 h at 37°C. Bacteria were removed by centrifugation and filtration and the resulting cell-free supernatants were assayed for lethal toxin activity by intravenous injection into Fisher 344 rats and edema-producing activity by intradermal injection into guinea pigs. All three parent strains exhibited both lethal and edema factor activities whereas the supernatants from the heat-treated strains possessed neither activity. Concentrated supernatant fluids from heat-treated and untreated cultures were also tested for soluble antigen by an Ouchterlony double-diffusion assay using guinea pig antisera developed against concentrated Weybridge supernantants containing crude whole toxin. With each parent strain supernatant, two strong percipitin lines were consistently seen. The heat-treated strains occasionally showed a single, barely discernible precipitin line, which might represent recognition of soluble, nontoxic antigen in the supernatants. When assayed using Coomassie blue, the protein concentrations of the culture supernatants were found to be substantially greater for the parent than for the heat-treated strains.

Anthrax cultures were screened for extrachromosomal DNA by a modified sarkosyl lysate technique. Plasmids were purified further by isopycnic ultracentrifugation on cesium chloride-ethidium bromide gradients and resolved by agarose gel electrophoresis. Single plasmid species detected in each untreated parent strain had

TABLE VIII. FORMULATION OF R MEDIUM

INGREDIENT	mg/LITER
Leucin	230
Histidine	55
Proline	42.5
Tryptophan	35
Phenylalanine	125
Glycine	65
Lysine	230
Arginine	125
Methionine	72.5
Isoleucine	170
Threonine	120
Serine	235
Valine	172.5
Tyrosine	144
Cystine	25
Thiamine	1.0
$MnSO_4$	0.90
$CaCl_2$,2 H_2O	7.4
$MgSO_4$,7 H_2O	9.9
Adenine Sulfate	2.1
Uracil	1.4
Sodium Glutamate	612
Aspartic Acid	184
$NaHCO_3$	8,000
K_2HPO_4	3,000

R medium ingredients were added as concentrated stock solutions, except for tyrosine, Glutamic acid Aspartic acid, K_2HPO_4 and $NaHCO_3$, which were added as solids. $NaHCO_3$ was added after all other regents were combined: medium was then filter-sterilized through A 0.45- μm filter. Medium was stored in the cold in tight capped flasks.

TABLE IX. PRODUCTION OF ANTIGENS IN VARIOUS MEDIA BY 3 STRAINS OF B. ANTHRACIS

Strain	Reciprocal Ouchterlony Titer						Toxic Units/ml Medium		
	1095		Thorne		R				
	PA	LF	PA	LF	PA	LF	1095	Thorne	R
Sterne	1	-	4	1	4	2	5	58	84
V770-NP1-R	1	-	1	-	4	1	23	25	79
Vollum 1B	1	-	8	2	8	2	15	143	202

molecular weights which ranged from 30-60 X 10^6. Plasmid DNA was not detected by cesium chloride centrifugation or gelelectrophoresis in cultures which had been passaged at 42.5°C. No reversion to the parent phenotype or toxin production was demonstrated when heat-treated isolates were regrown at 37°C for ten daily subcultures, nor were plasmid elements detected in these cultures.

Production of protective antigen, Lethal Factor and Holotoxin in 1095, Thorne's casamino acids and R media by 3 strains of B. anthracis was examined. PA and LF serologic activity was assayed by double-diffusion technique using antisera recognizing PA and LF. Holotoxin biologic activity was measured by the rat lethality assay.

In addition, we have demonstrated that a Pasteur vaccine strain of B. anthracis (American Type Culture Collection No. 4229) also does not contain plasmid elements. Furthermore, lethal toxin and edema-producing activities were not detectable in culture supernatants; this strain was also similar to the heat-treated strains with respect to serological activity and protein concentration in the supernatant.

Based on these observations, it was necessary to demonstrate that the elimination of plasmids was not a coincidental event to heat-attenuation of chromosomal-borne toxin genes. Transformation experiments were therefore performed according to the method of Chang and Cohen using heat-treated V-770-NP1-R cells and plasmid DNA purified from the parent strain. Four hundred fifty colonies from regeneration media plates were stab-inoculated onto immunoassay plates which contained the defined medium, 1% agarose and 1.5 ml of antiserum from goats (immunized with viable spores of the Weybridge strain). Parent and heat-treated isolates were used as controls on all assay plates. Three transformants were identified by immunoprecipitin halos which formed around the colonies. The supernatants from broth cultures of these transformants and from cultures of colonies which did not evidence precipitin halos were reassayed for edema-producing and lethal toxin activities. Cells were also screened from extrachromosomal DNA as previously described. Lethal toxin and edema-producing activities were restored and plasmid DNA, similar in molecular weight to that of the parent strain, was reisolated only from cultures of the three transformants.

It appears that plasmid genes are involved in the production of anthrax toxin, although their specific role remains to be elucidated. Additionally, it is likely that Pasteur's success in attenuating of the anthrax bacillus occurred as a result of curing the strain of its plasmid component through sequential passages at elevated temperature.

The application of recombinant DNA technology will greatly facilitate a better understanding, not only of the molecular interactions of each toxin moiety, but also of the mechanism of action of the anthrax holotoxin. Conceivably, these studies will eventually contribute to the development of a more efficacious human vaccine.

Electron microscopy of extrachromosomal DNA. The objectives of this portion of the research on Bacillus anthracis are 1) determine the size and structure of plasmids isolated from various strains of B.anthracis (i.e., molecular weight and circularity) and 2) analyze the homogenity between the plasmids of the various strains (heteroduplex mapping).

Direct observation of extrachromosomal DNA requires a reliable spreading technique. However, we were unable to obtain satisfactory results following published protocols (ammonium acetate and formamide techniques). In January, Dr. M. Gonda spent a day training us in the peculiarities of the formamide technique. Since then, we have established a protocol based on Dr. Gonda's methods that give satisfactory results based on observations from Col E1 and X174 circular DNA, Plasmids from V770 have been photographed and are awaiting measurement. Preliminary data by EM indicate that the molecular weight of V770 plasmid ($>$ 110 md) is much larger than originally estimated by gel electrophoresis (30-70 Md).

In vitro assays of virulence and host phagocytic defense system. Ongoing research is intended to determine the relationship between B. anthracis virulence and host phagocytic defense systems. Initial studies have involved exposing neutrophils in heparinized blood from anthrax-vaccine naive human donors to one of the following: radiation-killed encapsulated B. anthracis with and without specific antisera; B. anthracis crude toxin; lethal factor (LF); protective antigen (PA); or concentrated PA. The blood plus test material were contained in 10 X 75 mm serologic tubes incubated at 37°C for 1 h on a rack rotating 20 r.p.m. Giemsa-stained blood smears were prepared from test mixtures containing B. anthracis organisms and the percentage of neutrophils ingesting B. anthracis determined. Meanwhile, aliquots of 18 h culture of Vibrio parahemolyticus were added to the test mixtures which were then incubated another hour as described; Giemsa-stained blood smears were again prepared and the percentage of neutrophils ingesting V. parahemolyticus determined, and microscope slide cultures of each test mixture were prepared, incubated at 37°C for 3 h, and the percentage of neutrophils containing viable V. parahemolyticus determined.

Preliminary results indicated that: 1) radiation-killed encapsulated B. anthracis were very actively phagocytosed by all neutrophils in heparinized human whole blood, and the addition of specific capsular or cell wall rabbit antiserum to test system had no appreciable opsonic effect; 2) pre-treatment of heparinized whole blood with radiation-killed encapsulated B. anthracis, crude toxin, LF, or PA had no effect on the vibriocidal activity of human neutrophils ingesting V. parahemolyticus; 3) the effect of neutrophil ingestion of dead encapsulated B. anthracis on subsequent phagocytosis of V. parahemolyticus could not be determined because phagocytized B. anthracis obscured the ingested vibrios in many neutrophils. Pre-treatment with crude toxin or LF did cause a significant decrease in the percentage of

neutrophils ingesting V. parahemolyticus in half the blood samples; and pre-treatment with protective antigen almost completely inhibited phagocytosis in the samples tested.

These studies are continuing and will be expanded to include live encapsulated B. anthracis and macrophages as well as neutrophiles under in vivo conditions.

Presentations:

1. Stokes, W. S., and J. C. Donovan. Naturally occurring malaria in nonhuman primates. Presented, Operational Laboratory Animal Problems Course, Air Force School of Aerospace Medicine, Brooks Air Force Base, TX, 3-7 May 1982.

2. Stokes, W. S. Naturally occurring malaria in nonhuman primates: a review and clinical case report. Presented, USAMRIID Professional Staff Conference, Fort Detrick, MD, 29 April 1982.

3. Stokes, W. S. Diseases of laboratory animals. Presented, AALAS Animal Technicians Course, Fort Detrick, MD, 22 February 1982.

4. Ezzell, J. W. Chemical and immunological analysis of Bacillus anthracis cell walls. Presented, 82nd Annual American Society for Microbiology Meeting, Atlanta, GA, 7-12 March 1982.

5. Ezzell, J. W. Chemical composition analysis of Bacillus anthracis. Presented, Bacteriology Division Professional Staff Conference, USAMRIID, Fort Detrick, MD, 25 February 1982.

6. Mikesell, P., B. E. Ivins, and J. D. Ristroph. Evidence for plasmid-mediated toxin production in Bacillus anthracis. Presented, Regional Maryland and Washington, D.C. Branch American Society for Microbiology, USAMRIID, Fort Detrick, MD, 19 February 1982.

7. Mikesell, P., B. E. Ivins, and J. D. Ristroph. Plasmids, Pasteur, and Anthrax. Presented, Bacteriology Division Professional Staff Conference, Fort Detrick, MD, 25 February 1982.

8. Ivins, B. E., and J. D. Ristroph. The Medium is the Message: production of high levels of anthrax toxin under defined growth conditions. Presented, Bacteriology Division Professional Staff Conference, USAMRIID, Fort Detrick, MD, 25 February 1982.

9. Mikesell, P., T. Dreier, J. D. Ristroph, and G. B. Knudson. Plasmid isolation in virulent and avirulent strains of bacillus anthracis. Presented, 82nd Annual American Society for Microbiology Meeting, Atlanta, GA 7-12 March 1982.

10. Knudson, G. B., B. E. Ivins, and P. Mikesell. Plasmid-associated toxin production in Bacillus anthracis. Presented, 82nd Annual American Society for Microbiology meeting, Atlanta, GA, 7-12 March 1982.

11. Ristroph, J. D., and B. E. Ivins. Elaboration of Bacillus anthracis antigens in a new synthetic medium. Presented, 82nd Annual American Society for Microbiology Meeting, Atlanta, GA, 7-12 March 1982.

12. Hedlund, K. W., S. F. Little, D. W. Seburn, and R. A. Rotella. Isolation of protective antigen from anthrax toxin by preparative isotachophoresis. Presented, 3rd International Symposium on Isotachophoresis, Goslar, F.R.G., 1-4 June 1982.

Publications:

1. Hedlund, K. W., S. F. Little, D. W. Seburn, and R. A. Rotella. 1982. Isolation of protective antigen from anthrax toxin by preparative isotachophoresis. In: Analytical isotachophoresis, Holloway, C., (ed.). Proceedings of the 3rd International Symposium on Isotachophoresis, 1982, Elsevier Scientific Publishing Co., Amsterdam (in press).

2. Mikesell, P., B. E. Ivins, and J. D. Ristroph. 1983. Evidence for plasmid-mediated toxin production in Bacillus anthracis. Infect. Immun. 39:371-376.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
				DAOG 1537	82 09 30	
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
81 10 02	K. COMPLETED	U	U		NL	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	BC	148
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE *(Precede with Security Classification Code)*
(U) Prevention of Viral Diseases of Potential BW Importance

12. SUBJECT AREAS
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
80 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	35.2	2,650
c. TYPE	d. AMOUNT	83	0.0	0
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS *(include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR Peters, C J
d. TELEPHONE NUMBER *(Include area code)* 301-663-2833	d. TELEPHONE NUMBER *(Include area code)* 301-663-7244
21. GENERAL USE FIC MILITARY/CIVILIAN APPLICATION:	f. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Jahrling, P B g. NAME OF ASSOCIATE INVESTIGATOR *(If available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Viral Diseases; (U) Vaccines; (U) Laboratory Animals

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Study pathogenesis of selected toga-, bunya-, and arenaviruses unique in their capacity to cause widespread epidemics in military populations. Research focuses on identification of the determinants of viral disease. Understanding will allow development of attenuated and/or inactivated vaccines or therapeutic regimens for use in military personnel.

24. (U) Suitable animal models are developed, preferably using discrete genetic differences, to highlight key steps in pathogenesis. Viral replication, antigen expression, and immune responses are monitored during infection to allow inferences about critical determinants which may be tested by experimental manipulation.

25. (U) 8110 - 8209 - Efforts continue to emphasize lethal hemorrhagic fever viruses. A plaque assay was developed for Hantaan virus and was used to measure antibody in animal and convalescent human sera. Serum samples (385) were obtained recently from Liberian patients acutely ill with Lassa fever. Serologic testing indicated 87 seroconversions. Thirty-five potential donors of high-titered plasma have been identified and 21 units of immune plasma have been received and tested. Genetic resistance to RVF in the inbred rat was shown to be reflected in vitro by cultured macrophages. Macrophage resistance is not a function of the resting state of the cell but reflects the strength of the antiviral state induced by interferon. Pathogenesis studies of lethal Pichinde virus infection in strain 13 guinea pigs showed that the main causes of death were associated with pulmonary edema, metabolic acidoses, decreased cardiac output and drastic reduction of body weight. Publications: Tolixon 19:701-704, 1981. Fed. Proc. 41:1133, 1982. Infect. and Trum. 37:771-778, 1982. Am. J. Trop. Med. Hyg. 31:1038-1045, 1982. J. Egpt. Pu. H. Assoc. 56:374-402, 1981.

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)

Work Unit No. 871 BC 148: Prevention of Viral Diseases of Potential BW Importance

Background:

This study is targeted toward the development of safe and effective vaccines to protect the soldier against certain unusual, highly dangerous viral infections such as Lassa, Ebola, Rift Valley fevers, Chikungunya infection, as well as Argentine, Bolivian and Korean hemorrhagic fevers. Except for a formalin-inactivated Rift Valley fever vaccine, acceptable vaccines are not as yet available. Specific antiviral therapy has been lacking and patients with any of these infections must be treated with general supportive measures, none of which are able to destroy the invading viruses. These viruses have characteristics of aerosol stability and high infectivity which make them significant threats as potential biological warfare (BW) agents. Studies on these highly dangerous viruses are time consuming because they must be performed in biohazard containment laboratories under the most rigid requirements for microbiological safety

These viruses are not only highly dangerous, but are poorly understood. It has been necessary, therefore, to develop a base of scientific information on which to make rational judgements regarding the methods for developing vaccines. This has necessitated the careful characterization of appropriate animal models in order to derive an enhanced understanding of disease pathogenesis, i.e., the nature and progression of the viral disease under study. Animal studies include identification of target organs and documentation of histopathological evidence of viral replication and of cellular injury; establishing the role of host immunity in protecting against, or contributing to, the disease process; identifying the role of host genetics in susceptibility and resistance; establishing the mechanisms of immunity to disease; determining the differential immune response to peripheral virus inoculation in comparison to aerosol challenge; and identifying significant factors for maintaining long-term immunity to these biological warfare threats.

The experimental approaches employed to achieve program objectives are multidisciplinary. They are based on innovative combinations of virology, immunology, serology, microbiology, immunochemistry, aerobiology, pathology, cellular biology, genetics and tissue culture technology.

The Principal Investigator for this work unit is LTC C. J. Peters, MC. Contributing research investigators and their area of research include: F. E. Cole and J. Barrera-Oro - Argentine hemorrhagic fever vaccine studies; F. E. Cole and N. H. Levitt - Chikungunya virus vaccine studies; P. B. Jahrling - Lassa fever studies; E. D. Johnson - Ebola virus studies; C. T. Liu - physiological aspects of the pathogenesis of arenovirus infections; C. J. Peters - Rift Valley fever virus genetic studies; D. D. LaBarre - Rift Valley fever immunological studies.

Progress:

Studies have continued on the development of an attenuated Argentine Hemorrhagic Fever (AHF) vaccine. The disease is acute, severe, moderately lethal and is caused by Junin virus, a member of the Arenavirus family. Since this virus is perceived to have significant BW potential, it is imperative that a safe and effective vaccine be developed in order to immunize troops against the disease.

As described in the FY 81 Annual report, three vaccine seed candidates were selected for further evaluation. All three candidate seeds were more attenuated than even the clone 3 vaccine, the strain used to immunize 636 people in Argentina in 1969 - 1971. This observation was based on results obtained by the intracerebral inoculation of 11-to-12-day old suckling mice and baby and adult guinea pigs. These candidate seeds were all shown to be genetically stable during multiple cell culture passage. One of them, Candidate #1, also demonstrated little or no neurovirulence as tested in guinea pigs and a few monkeys. This seed virus, Candidate #1, was used to prepare a secondary or production seed lot of 1.5 liters. The secondary seed was used to prepare a small lot of vaccine. The seeds and vaccine were produced under FDA Regulations [1,2] and are currently undergoing extensive safety testing. The results, to date, indicate no contamination and no loss of attenuation. A vaccine suitable for use in man should be available by late winter 1983, barring unforeseen circumstances.

Efforts to prepare a safe and effective vaccine for Chikungunya virus have not been successful due to a variety of problems; for example, failure of selected clones to maintain genetic stability and lack of immunogenicity. Careful examination of CHIK vaccine strain 181 revealed two sub-populations of virus plaque formers; a small plaque of 0.5 to 1.0 mm, and a medium sized plaque of 1.0 to 2.0 mm. The wild parent has a plaque size of 5 to 9 mm. Six small and six medium sized plaques were selected for additional study. Two are currently undergoing testing, one small plaque, termed clone 19, and one medium size plaque, termed clone 30. The other 10 clones are stored frozen as reserve seeds pending the results from current studies. Clones 19 and 30 were selected because of their acceptable virus titer, homogeneous plaque population and small and medium sized plaques, respectively.

In a plaque reduction neutralization test, 100 PFU of each clone (#19 and # 30) were completely neutralized by certified NIH Research Reference Reagent CHIK hyperimmune ascitic fluid, thus confirming their identity as CHIK viruses.

In vitro reversion studies were conducted with the two candidate clones to determine whether they are genetically stable; that is, would retain their characteristic biological markers of attenuation. This was accomplished by passing each clone for four serial passages in MRC-5 cells using high (undiluted) virus inputs, examining each passage level for observable changes in plaque size and titer.

Clone 19 increases in titer at each passage level with a concomitant increase in plaque size equal to that of the medium size plaque variant, clone 30. On the other hand, clone 30 maintains its titer throughout the four passage levels with a relatively slight increase in plaque size. Most importantly, the medium plaque variant does not revert after four passes to a large plaque size. Neurovirulence studies, in weanling mice and rhesus monkeys will be initiated shortly.

A total of 385 sera, obtained from acutely-ill, febrile patients hospitalized in Liberia, were tested for infectious virus, fluorescent, and neutralizing antibodies to Lassa virus. Twenty-seven fresh isolates were obtained, including two from patients who acquired their infections in Nigeria, and three from Guinea. Of the 385 sera, 87 titered 10 by indirect fluorscent antibody titer (IFAT) to lassa, but only 8 contained detectable neutralizing antibody. In a series of 10 patients from whom sequential (followup) sera were available, it is clear that while IFAT titers peak within one month of infection, neutralizing antibodies evolve more slowly, even when IFAT titers begin to wane. Thus, convalescent patients selected months to years after infection generally have higher log neutralization index (LNI) titers than those more recently infected. Higher LNI titers are also obtained when the geographic origins of plasma and challenge virus are matched. Neutralizing and IFAT responses in cynomolgus and rhesus monkeys surviving infection without therapeutic intervention were similar to the patterns observed in human patients. IFAT titers peaked by 30-60 days, then gradually receded, while LNI titers continued to increase for at least 8 months.

Higher quality human immune plasma units, that is those with higher LNI titers, were obtained once it was recognized that late convalescent plasma generally contained higher LNI titers. While plasma collected prior to 1981 was selected primarily on the basis of IFAT titers or even anecdotal information, plasma collected more recently was obtained primarily from patients with confirmed Lassa virus infections occurring 6 months or more previously.

Serologic relationships among Lassa virus strains were explored and compared to lymphocytic choriomeningitis (LCM) virus using the now standard virus dilution neutralization test, and a newly-developed serum dilution neutralization test enhanced by the addition of protein A. Using both procedures, isolates were serologically related, yet distinguishable. LCM was weakly neutralized by all Lassa strain antisera except Mozambique, yet LCM antiserum neutralized only LCM. Although correlation between serologic relationships and cross protection must await completion of current tests, LCM is clearly more closely related to Lassa virus strains than was previously surmised.

Experimentally inoculated primates are a potential source of high titered plasma and despeciation techniques could conceivably be employed to produce immune material suitable for use in human patients. An alternative approach is the fractionation and concentration of IgG from whole plasma using established techniques. Human and monkey immune plasmas were fractionated and concentrated. The human plasma, LNI of 0.6, did not protect guinea pigs, but following fractionation and concentration the undiluted material, with an LNI of 4.3 and 1:4 dilution, with an LNI of 2.4, both protected guinea pigs. Likewise IgG prepared from monkey plasma, with an LNI of > 4.3 at a 1:8 dilution, conferred protection to guinea pigs even when diluted 1:64. In another study, serologic differences among Lassa virus strains were defined in cross neutralization tests using guinea pig, monkey, and human convalescent plasma. Increased LNI titers, and enhanced protection of passively administered plasma, were obtained when geographic origins of virus and plasma were matched. It may be concluded from these discoveries that a solid foundation of knowledge is being generated from which better treatment modalities are becoming available for Lassa fever.

Ebola hemorrhagic fever virus (EHFV) studies have concentrated upon establishing insight into the epidemiology/ecology and pathogenesis of EHFV. Current investigations utilize the limited available techniques, while developing the necessary and additional tools; that is, assay procedures and experimental models, in order to explore both of these areas in greater depth. Along these lines, experiments have been undertaken: (1) to develop a reliable, sensitive assay for detecting the virus and circulating neutralizing antibody, as well as (2) to establish an animal model in which the importance of the host defense system to EHFV can be evaluated.

The observation that a significant proportion of guinea pigs being maintained as an additional food source by the natives of northwestern Zaire were seropositive to EHFV was the first indication that enzootic infections may occur in central Africa. An expedition was sent to this region in an attempt to better define the epidemiology/ecology and pathogenicity of EHFV. Field samples were collected from two areas and included sera from guinea pigs, rats and humans near households which maintained guinea pigs. Serum samples were screened by indirect immunofluorescents antibody test (IFAT) for antibody specific to Rift Valley fever virus, Lassa virus, Congo-Crimean hemorrhagic fever virus and Marburg and Ebola viruses. Rodent tissue and arthropod samples were also collected and screened for infectious Ebola virus. None of the samples were seropositive to Rift Valley fever or Lassa viruses; however, a low frequency of samples was found to be positive to Ebola, Marburg and Congo-Crimean hemorrhagic fever viruses. EHFV-positive guinea pig samples were collected in only three of six villages while seropositive natives were identified in all of the surveyed villages.

The epidemiological significance of circulating non-neutralizing IFAT detectable antibody in the native and guinea pig population of northwestern Zaire is unclear. The possibility that this antibody reflects either the presence of a less virulent virus which cross-reacts serologically with members of the Marburg/Ebola virus group or the true low pathogenicity of EHFV infections cannot be determined. In addition, the role that guinea pigs play in the maintenance and transmission cycles of EHFV is not yet clear. In light of the very preliminary observation that fatal infections can be transmitted orally to naive animals by the urine from infected animals, the possibility that rodents are in some way important in the dissemination of virus cannot be ruled out. The recent finding that mastomys and paomys captured in the Central African Republic are seropositive to EHFV may support this possibility.

Several techniques have been employed to develop an assay to measure infectious EHFV and neutralizing antibody. The possibility of using a plaque assay for this purpose was explored first using several cell lines, diluents, overlay media and various additives such as dimethylsulphoxide, protamine sulfate, DEAE dextran, actinomycin D and other variables. EHFV plaques best but inconsistently in Vero cells maintained in Eagles basal medium with Earle's salts, 0.4% agarose and 4% heat inactivated fetal calf serum. In addition, limited success has been achieved in establishing a stable plaque purified cytocidal virus clone which could be used in a plaque assay. However, the most promising technique to measure infectious virus has been an indirect radio-labelled antibody (RLA) method. This assay capitalizes on the consistent observation that infectivity as measured by immunofluorescence $TCID_{50}$ (50% tissue culture infectious dose) is consistently 100 times higher than that measured by a plaque assay.

At present, the ^{125}I-Staph-A system seems to be the most reliable and least time consuming method for detecting bound antibody. The enzyme systems are more sensitive, but unfortunately background noise has been routinely elevated. Procedures to reduce background reactions have failed to completely eliminate this problem. Incorporating alkaline phosphate labeled Staph-A into the assay may prove to be the best compromise. By utilizing the ^{125}I-Staph-A method, very low levels of infectious virus can be detected in MRXC-5 cells as early as 3 days postinfection.

Interferon may be an important factor in the recovery of EHFV infection because: (a) a potentially fatal human case was successfully treated with passively administered human leukocyte interferon and convalescent sera; (b) guinea pigs treated with the interferon inducer, Poly(ILLC) are resistant to a fatal EHFV challenge. Efforts to develop a guinea pig model to study interferon were not successful. The guinea pig is simply not an appropriate host. At present, it is unclear whether other laboratory animals, including the monkey, can be used to study interferon. Much more research needs to be performed on this highly dangerous but poorly understood virus.

Physiological studies of the pathogenesis of arenavirus infections were continued under P2 biohazard containment using Pichinde virus in strain 2 or 13 inbred guinea pigs and MHA strain of hamsters. The advantages for using Pichinde virus are obvious: the virus does not produce any adverse effects in man; the symptoms produced in the inbred guinea pig closely parallel those produced by the more virulent arenaviruses. Pathogenesis studies of lethal Pichinde virus infection in strain 13 guinea pigs were emphasized. The most striking results indicate that the main cause of death were associated with pulmonary edema, metabolic acidosis, vasoconstriction, decreased cardiac output, and a drastic reduction of body weight. Balance studies for energy, water, nitrogen, fat and electrolytes were also initiated and techniques were established for collecting urine and feces using the individual pan with modifications. Total body composition of the guinea pig was achieved by grinding the dessicated body into a fine homogenous powder for extraction and dissolution prior to chemical analyses. X-ray techniques for taking a clear chest picture were standardized in order to visualize pulmonary edema and heart size of the guinea pig. Moreover, a tilt-table was constructed for studying orthostasis and integrity of the sympathetic nervous systems in maintaining blood pressure during tilting. This will reveal early compensated cardiovascular dysfunctions during infection. Finally the physiology laboratory was returned from USAMBRDL to the new section of building 1412.

One aspect of this research continues to be the understanding of the genetic basis of resistance to fulminant RVF infection in a defined rat model. It was previously reported that macrophage cultures *in vitro* reflected the different *in vivo* susceptibility of resistant Lewis (LEW) and susceptible Wistar Furth (WF) rats. These observations were extended to show that virus yield is greater and the amount of virus necessary to infect is less in WF that LEW macrophage cultures. These findings suggest that one mechanism which controls liver virus replication in LEW rats is the difficulty in infecting liver macrophages and the small amount of progeny virus obtained from those phagocytes which are infected. Macrophage resistance appeared to be due to the strength of the antiviral state induced by interferon or other lymphokines.

The observation of large differences in susceptibility of certain inbred rat strains to the ZH501 strain of RVFV was made more interesting by the finding that only ZH501 and other Egyptian virus strains showed this behavior. RVFV strains from Kenya, Uganda, Zimbabwe, and South Africa were virulent for other animals but did not kill rats even of a susceptible genotype. This finding of selective susceptibility, manifest only with a precise host-virus combination, is an important concept in viewing pathogenesis of viral diseases and inferring whether or not a virus is "virulent". In the combination using RVF and rats, the Egyptian strains such as ZH501 are only highly virulent in a particular adult inbred rat line. In general, there is no clear evidence that Egyptian viruses are "hot" or that the WF rat is more susceptible to infections. Additional evidence to support this concept was obtained with Phlebovirus systems under study. Last year it was reported that eastern Panamanian strains of Punta Toro (PT) virus, such as the Adames strain (PT-A), were virulent for adult hamsters whereas other strains such as the prototype (PT-P) were not. Additional surveys were completed and it was discovered that Chinese hamsters (Cricetulus griseus) succumb to infection with PT-A but not PT-P. It was also shown that cotton rats (Sigmodon hispidus) from our colony are resistant to ZH501 but that NIH cotton rats are exquisitely susceptible. The Mexican-derived cotton rat colony interbreeds with the NIH US-derived colony, confirming that they are the same species and allowing future studies to determine the mode of inheritance.

The interferon inducing ability of CP20,961 has been examined in cynomolgus monkeys at 0.1%, 1.0, and 10 mg/kg dose levels in combination with RVF vaccine NDBR-103 lot 3. Plasma interferon levels were significantly elevated in the high dose group for 4 weeks postvaccination, while the other groups showed only a transient peek at 48 hours postvaccination. Moreover, the adjuvant enhanced lymphocyte transformation in RVF vaccinated monkeys.

Cellular cytotoxicity and lymphocyte transformation assays have been refined and used to assess human, rhesus and cynomolgus monkeys immunized with RVF vaccine. Circulating T-lymphocytes were demonstrated in all vaccinates, and cytotoxicity seems to be necessary for in vitro blast transformation to RVF antigen. These data indicate a significant T-cell component in the cellular response to RVF vaccine.

Important collaborative RVF research projects are ongoing with results briefly as follows:

1) Virus isolates, thought to be attenuated by Dr. I. Z. Imam, Agouza, Vaccine Institute, Egypt, have been received and shown to be RVFV. They are fully virulent for adult hamsters and mice and do not show any evidence of attenuation. Dr. Ahmed El Kafrawi, also from Egypt, has been working in our laboratory with several rodent isolates from Egypt. His material appears to contain one or more agents which are morphologically or serologically related to Phleboviruses.

2) The ongoing human vaccine trail with Dr. Jeremy Kark, now at Hadassah Medical School, Jerusalem, Israel, has now entered the 18 month booster stage and serological data are being analyzed at this time.

3) Dr. Niklasson, State Bacteriological Laboratory, Stockholm, appears to have both a viable antigen and antibody ELISA for RVF. Antibodies have not been tested for cross-reactivity but false positives do not seem to be a problem.

4) Dr. James Meegan (YARU) has produced a dozen monoclonal antibodies to RVFV, most of which we found to have neutralizing capability and Dr. Joel Dalrymple, of USAMRIID, has a larger bank of antibodies which do not neutralize viral infectivity. These monoclones have been tested against several different virus strains and at least one clearly reacts with Egyptian virus but not with Ugandan, Kenyan, Zimbawean, or South African RVF strains.

5) Dr. A. Antoniades from the U of Thessalonika, Thessalonika, Greece, provided our laboratory with sera from persons living in Greece who had HAI antibodies to RVFV in low titer. We tested there in RVFV specific PRN and they were negative suggesting they were a result of cross-reacting phlebovirus infection. The sera were also tested for fluorescent antibodies to Korean Hemorrhagic fever and showed that 2 of 108 were positive in reasonable titer.

6) Dr. Genovesi, an NRC postdoctoral student in our laboratory, is developing a fatal LCM model in hamsters. He has cloned the WE strain and identified both attenuated and lethal substrains. In the lethal model, death occurs about 3 weeks post inoculation and is associated with artheritis. Weight loss is profound. Studies of virus replication, antibody formation, and immune complexes are underway.

7) Dr. J. P. Gonzalez (now at CDC, Atlanta, formerly Institute Pasteur Banqui) provided human sera from the Central African Republic, and high titered PRN antibodies to RVFV were detected in about 15% of them. Previous reports of HAI antibodies in sheep and Dr. Gonzales' findings of 7% IFA antibodies in the human sera raised the possibility of RVF existing in that country; however, the HAI and IFA tests are too cross-reactive to be definitive. These sera were tested for PRN antibodies to other known African phleboviruses and all except RVFV are negative, confirming the RVFV specificity.

Presentations:

1. Jahrling, P. B. and C. J. Peters. Beneficial and harmful effect as Lassa-immune responses in primates (invited symposium). Am. Soc. Trop. Med. Hyg., Annual Meeting, San Juan, P.R., November 1981.

2. Jahrling, P. B. Association of variables in Immunotherapy of Lassa virus infection of animal models. American Society for Virology, Charter Meeting, Ithaca, New York, August 1982.

3. Peters, C. J. and G. W. Anderson. Resistance to Phleboviruses. Presented US-Japan Cooperative Medical Science Program, Bethesda, MD, 9 November 1981.

4. Peters, C. J. and G. W. Anderson. Pathogenesis of Phlebovirus Infections, Presented 30th Annual Meeting, Am. Soc. of Trop. Med. and Hyg. San Juan, P.R., November 1981.

5. Peters, C. J. Agar Gel Precipitin Test for Rift Valley Fever Diagnosis. Presented MZCCI HPI/WHO Workshop on Rift Valley Fever Seroepidemiology and Diagnosis, Athens, Greece, 26-30 April 1982.

6. Peters, C. J., H. Schellekins, J. A. Rosebrock and G W. Anderson. Genes, Macrophages, and Resistance to Rift Valley Fever in the Rat. Presented, First Annual Meeting, Am. Soc. for Virology, Ithaca, NY, August 1982.

7. Peters, C. J. Rift Valley Fever: Immunology and Genetics. Immunology Research Conference, University of North Carolina, Chapel Hill. 25 February 1982.

8. Peters, C. J. Pathogenesis and Epidemiology of Hemorrhagic Fevers in Pathology 17, John Hopkins University School of Hygiene and Public Health, Baltimore, MD, 16 April 82.

9. Peters, C. J. Rift Valley and Congo-Crimean Hemorrhagic Fevers, in Global Medicine Course, School of Aerospace Medicine, San Antonio, TX, 13 April 1982.

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11. Peters, C. J. Defensive Concepts Concerning Viral Diseases, in Medical Defense Against BW and Highly Communicable Infectious Agents, USAMRIID, June 1982.

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1. Jahrling, P. B., Smith, S., Hesse, R. A., and Rhoderick, J. B. 1982. Pathogenesis of Lassa Virus infection in guinea pigs. Infect. Immun. 37:771-778.

2. Callis, R. T., Jahrling, P. B., and DePaoli, A. 1982. Pathology of Lassa virus infection in the rhesus monkey. Am. J. Trop. Med. Hyg. 31:1038-1045.

3. Liu, C. T. and E. J. Galloway. 1981. Changes in tissue cyclic AMP concentrations following an intravenous lethal dose of cholera enterotoxin in rabbits. Toxicon 19:701-704.

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7. Peters, C. J. Phagocytosis and cytotoxicity by murine macrophage cell lines. J. Reticuloendothelial Soc., submitted for publication, 1982.-

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2. Food and Drug Administration. 1981. Gerneral pp. 209-223. Code of Federal Regulations, Title 21, Chapter 1, Subchapter A. U.S. Government Printing Office, Washington, DC.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
				DAOB 6420	81 10 01	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
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10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	BD	147
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE *(Precede with Security Classification Code)*
(U) Evaluation of Experimental Vaccines in Man for BW Defense

12. SUBJECT AREAS
003500 Clinical Medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
61 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	1.1	395
c. TYPE	d. AMOUNT	83	6.0	426
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Medical Division, USAMRIID
b. ADDRESS *(include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR Peters, C J
d. TELEPHONE NUMBER *(include area code)* 301-663-2833	d. TELEPHONE NUMBER *(include area code)* 301-663-7655
21. GENERAL USE FIC	f. NAME OF ASSOCIATE INVESTIGATOR *(if available)*
MILITARY/CIVILIAN APPLICATION:	g. NAME OF ASSOCIATE INVESTIGATOR *(if available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Vaccines; (U) Prophylaxis; (U) Therapy; (U) Infectious Diseases; (U) Human Volunteers

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Evaluate experimental vaccines developed by USAMRIID, various contractors, organizations or other governmental agencies. Assess effect of antimicrobials, various drug regimens and immune plasma in treatment of militarily important infectious diseases. This work unit is an essential element in a comprehensive program for medical defense against BW agents and other infections of unique military importance by allowing testing in man of newly developed experimental vaccines, new drugs for chemoprophylaxis or therapy, and immune plasma for globulins.

24. (U) Test vaccines, experimental drugs and newly developed hyperimmune plasma and/or globulins are given to human volunteers after both full safety testing in animal models and approval under strict protocol conditions which have undergone evaluation by scientific and medical ethics reviews.

25. (U) 8110 - 8209 - Preparations for testing Rift Valley fever vaccine (TSI-GSD-200) and determination of dose-response curves are underway. A protocol comparing the reactogenicity and immunogenicity of the Parke-Davis pentavalent botulinum toxoid and the Michigan Department of Public Health (MDPH) monovalent B toxoid was completed as was immunization of USAMRIID volunteers with MDPH type E monovalent toxoid. The program for the collection of botulism immune single donor plasma (human) IND-1332 was completed. Phase II trials using the antimalarial halofantrin (WR 171,669) continued. The Ward's high containment isolation suites underwent remodeling to upgrade them to P-4 isolation capabilities. They were used after a possible accidental exposure to Ebola virus. USAMRIID's Medical Isolation Team conducted 2 aeromedical evacuation exercises with the Air Force. In-house training exercises of the isolation facilities continued.

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)

Work Unit No. 871-BD-147: Evaluation of Experimental Prophylactic and Therapeutic Regimens in Man

Background:

On 1 November 1981 a fifth department was designated in the Medical Division, the Department of Occupational Medicine. It consists of a physician and an occupational health nurse. The primary function of this new department is to operate the special immunizations program to administer both experimental and licensed vaccines to personnel at risk of exposure to selected infectious agents and to monitor their serologic response. A primary objective is to insure that data collected are readily available for analysis and interpretation. Some immunization data have been placed in computer storage via punch cards since 1973. However, these data have not been formatted for optimal retrieval, nor are all the data forming a given individual's complete immunization record computerized. This limits analysis to an inefficient combination of manual and computer manipulations. Moreover, data on at least two vaccines being used have never been computerized. A second objective is to analyze systematically vaccine by vaccine, the enormous amount of data collected over the years. It is through this mechanism that recommendations for modifying the approach to immunization evolves. In conjunction with this effort, an expanded evaluation of the immune response becomes a clear obligation in order to understand better the efficacy of immunization. A third objective is to develop a computerized health profile system which links the immunizations history with the remainder of the medical history, especially that acquired by the Fort Detrick Occupational Health Program.

Progress:

This has been a year, not for research, but for establishing an efficient special immunizations program. Preliminary examination of some of the collected immunization data has uncovered several areas for fruitful research, however. Following the acquisition of a computer terminal dedicated to special immunizations program use, it was possible to examine some of the data in the data bank recorded for EE, WEE, and VEE vaccines. From this analysis, it was apparent that many individuals have a "flat" response to these vaccines as currently administered. This realization led first to a reconsideration by the USAMRIID Special Immunizations Committee of the spectrum of need for individuals to have EEE and WEE vaccines and secondly, to a revision of the scheme for administering VEE vaccine. It is planned that the response to these three vaccines will be closely monitored in new vaccines. Moreover, individuals who have essentially not responded to VEE vaccine in the past, at least by current criteria, will be reimmunized, following this scheme, and likewise closely monitored for response. The results will be reported in detail in a subsequent report.

As a result of a review of data on the use of anthrax vaccine at Dugway Proving Ground, UT, and its comparison with a limited amount of data from USAMRIID personnel given this vaccine, differences were observed which raise questions as to why two such groups would not respond similarly. These observations have opened the way to acquiring and computerizing all the data on anthrax vaccine use, as well as Botulinum Toxoid Vaccine use, and extending these analyses.

With the arrival of a new Director of Facilities Engineering, Fort Detrick, came a concern for the immunization status of his employees who service USAMRIID. The review necessitated by this concern had to be conducted manually, record by record, emphasizing the need for a computerized individual profile. This step will follow the data entry program. In the interim, however, a master roster including some of the more essential data was created and computerized, giving immediate access to the data required to determine the immunization status of an individual, or a group. A similar roster being developed for USAMRIID personnel will permit advising division chiefs at frequent intervals of the immunization status of personnel under their supervision.

The USAMRIID tuberculosis surveillance program, utilizing tuberculin testing, had lapsed about two years ago when personnel changes took place. In the past year, with the reorganization of the Medical Division and the development of the Fort Detrick Occupational Health Program, tuberculin testing was reinstated. As a cooperative effort, all available tuberculin testing data were computerized in the USAMRIID special immunizations office for use by the Fort Detrick occupational health nurse as required. New test results are added to these data to maintain a current status roster of USAMRIID personnel.

Significant Achievements:

1. Acquired a computer terminal and printer in the special immunizations office for direct linkage to the USAMRIID DEC PDP-11 minicomputer and to the Fort Detrick IBM 360 mainframe computer.

2. Began a systematic preliminary review of poor responses to VEE Vaccine for presentation to the Commander and a select committee for resolution.

3. Wrote the necessary computer software to track vaccine participants and record their data in a modified VEE immunization scheme.

4. Defined requirements for additional divisional ADP hardware to increase the level of computer interaction for data management, especially in occupational medicine.

5. Initiated action to incorporate previously inaccessible serologic data for anthrax and botulinum toxoid vaccines into the principal computer data bank.

6. Initiated and completed a computerized master roster of Fort Detrick Facilities Engineering personnel active in the special immunizations program.

7. Initiated a computerized master roster of USAMRIID personnel active in the special immunizations program.

8. Reinstated the USAMRIID tuberculosis surveillance program (tuberculin testing) and computerized the available historical data.

9. Finalized a revised data entry software program to allow direct entry of immunization data from the special immunizations office.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
				DAOG 3815	82 10 01	
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISS'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
81 10 01	D. CHANGE	0	U		NL	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M162770A871	BE	146
b. CONTRIBUTING				
c. CONTRIBUTING	STOC 80-7.2:2			

11. TITLE *(Precede with Security Classification Code)*
(U) Exploratory Antiviral Drug Development

12. SUBJECT AREAS
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology; 012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 10	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	13.2	653
c. TYPE	d. AMOUNT	83	14	995
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS *(Include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR Canonico, P G
d. TELEPHONE NUMBER *(Include area code)* 301-663-2833	d. TELEPHONE NUMBER *(Include area code)* 301-663-7244
21. GENERAL USE FIC MILITARY/CIVILIAN APPLICATION:	f. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Huggins, J g. NAME OF ASSOCIATE INVESTIGATOR *(If available)* Linden, C L

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Antiviral Drugs; (U) Pharmacology; (U) Viral Diseases; (U) Laboratory Animals

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Identify effective drugs against viruses that are potential threats to military personnel; obtain data on toxicology, pharmacology, and metabolism of antiviral drugs and conduct preclinical and clinical studies to assess safety and efficacy in compliance with FDA regulations; conduct laboratory studies to develop novel antiviral drugs by identifying potential targets for pharmacologic intervention.

24. (U) Assess efficacy of potential antivirals against viruses in tissue cultures and in rodent models for Rift Valley fever (RVF) and VEE. Evaluate toxicity and pharmacology of promising compounds in preclinical protocols conducted in rodents and nonhuman primates. Describe mechanisms of action and metabolism of new drugs and provide analytical support for drug analysis. Apply approaches of molecular virology and cell biology to define the molecular basis of virus-cell interactions.

25. (U) 8110 - 8209 - Seventy nucleotide and nucleoside derivatives, six drugs with reported in vitro antiviral activity and four antiviral drugs in combination with ribavirin, were evaluated for efficacy against 5 viruses in tissue culture. All but sixteen of the compounds tested showed some antiviral activity to one or more of the test viruses. Tests in mice with ribavirin in combination with Nafenopin, Clofibrate, 9-(2,3-hydroxypropyl-monohydrate) adenine or 9-(tetra-hydro-2-furyl) Purine 6-thiol showed no enhancement of antiviral activity against RVF virus over ribavirin alone. Ribavirin was shown not to alter the deformability of erythrocytes as measured by the ability of ribavirin-treated cells to pass through a 3 micron pore filter. The internalization of vesicular stomatitis virus was shown to be mediated through intermediate (non-lysosomal) vesicles. Didemnin A, a depsipeptide, was shown to be a potent inhibitor of protein synthesis. Its inhibitory dose (ID-50) was found to be 2.2 micrograms per ml.
Publications: Fed Proc. 40:634, 1981; 2 chapters in "Infection", 1981.

BODY OF REPORT

Project No. 3M162770A871: Military Disease, Injury and Health Hazards (U)

Work Unit No. 871 BE 146: Exploratory Antiviral Drug Development

Background:

Vaccine prophylaxis is not effective for the prevention of all virus-induced diseases. Vaccines also are usually virus-specific and ineffective after the onset of infection.[1] Chemotherapeutic agents are critically needed for the prevention and treatment of virus induced diseases.[2] Thus, a program was established in 1980 for the primary evaluation of promising antiviral drugs. This program evaluated drugs *in vitro* and in those instances where sufficient quantities were available, *in vivo*. In October 1980 the drug screening program was placed under a USAMRIID contract to Swiftwater Laboratories. In-house evaluations continued, however, while waiting for the contractor to establish operations.

The further evaluation of the antiviral agent ribavirin has been a prime focus of the work unit for the last year. Studies have moved beyond preliminary antiviral *in vitro* and *in vivo* evaluation of antiviral efficacy to an extended study of ribavirin's toxicity and chemotherapeutic capabilities in animal studies. Such animal studies have moved from rodent screens into primate models.

In support of USAMRIID's exploratory antiviral drug development, fundamental studies were carried out to elucidate the mechanism of action of antiviral agents, investigate the cellular basis of their toxicity, contribute to the evaluation of structure/activity relationships and discover cellular targets of opportunity for therapeutic intervention with antiviral agents. Specifically, studies were carried out to determine if ribavirin alters the deformability of red cells. This research represents our continued efforts to fully assess the toxicological effects of this antiviral agent. In order to establish a more rational approach to future drug design, cellular biochemical effects are to be evaluated for all drugs tested *in vitro* so that structure/activity relationships may be established and used to direct the synthesis of new compounds. For these studies, an approach is being developed to determine drug effects on cellular nucleic acid metabolism. Other areas of fundamental research initiated within the scope of this work unit include studies on viral uncoating and processing, role of lysosomal enzymes on degradation of viral proteins and mechanism of action of didemnins, a novel group of polypeptides with antiviral activity.

Drug Screening

Ribavirin analogs and other antiviral drugs were screened and evaluated *in vitro* against RVF, VEE, PIC, YF, SFS and VS viruses (VSV was added to the screen during this report period). *In vivo* studies were done in mice against RVF and in guinea pigs against PIC viruses. The results of the *in vitro* evaluations are shown in Table I.

Sixteen of the compounds did not show antiviral activity against any of the test viruses *in vitro*. Most of the drugs, however, did show some detectable activity to one or more of the test viruses.

Two compounds, didemnin A and B, (a new class of depsipeptides, isolated from a Caribbean tunicate)[3], showed antiviral activity against all of the test viruses at concentration ranging from 0.25 to 5.0 µg/ml in vitro. Mouse studies with RVF virus, showed that treatment with didemnin B, 0-25 mg/kg/day, resulted in 90% survival. Didemnin A at 1.25 to 5 mg/kg/day increased survival of mice by 50%. On an extended schedule, treatment of mice over a nine day period with didemnin B did not enhance the survival rate. Didemnin B was toxic and uniformly lethal to mice when administered at 1.0 mg/kg/day for 5 days.

Drug LY-122771/2 (Lilly) was retested in an expanded study to confirm the results reported by Swiftwater where all mice treated with this drug survived a challenge with RVF virus. Initial tests performed in our lab had shown some antiviral activity in vitro but no protection in vivo against RVF virus. The retests in our lab confirmed our original observations.

Ribavirin-Guanosine Treatment of Rhesus Monkeys. Twelve adult rhesus monkeys (Maccaca mulatta) which had not received ribavirin in the previous year were randomly assigned to each of three groups. Group I received ribavirin, 60 mg/kg/day, injected intramuscularly (IM) in two 30 mg/kg doses given at 12-hour intervals for 10 days. Group II received guanosine monophosphate (GMP), 250 mg/kg/day, injected IM in two 125 mg/kg doses given at 12-hour intervals for 10 days. Group III received 250 mg/kg/day GMP and 60 mg/kg/day ribavirin on the same schedule as groups I and II.

Blood samples were obtained from each monkey on days -7, -4, 0, 3, 7, 10, 14, 17, 21, 28, and 35. Samples were analyzed for hemoglobin concentration, packed red cell volume (Hct), red blood cell count (RBC), platelet count, white blood cell count (WBC), differential count, reticulocyte count, and uric acid levels.

In both Group I (ribavirin) and Group III (ribavirin and GMP), red cell counts reached a low on day 14. In the ribavirin-treated group the pretreatment count of 5.68 million RBC/dl fell to 2.86 million on day 14. In the ribavirin-GMP-treated group a pretreatment count of 5.97 million RBC/dl dropped to 2.92 million by day 14. The hemoglobin and hematocrit response in both groups followed the same pattern as the RBC count. Reticulocyte response to the anemia was not observed until day 14, five days after treatment was terminated, and reached a peak on day 28 in both groups I and III. In both the ribavirin group and ribavirin-GMP group a striking thrombocytosis was induced, with baseline values of 309,000 for the ribavirin group and 370,000 for the ribavirin-GMP group reaching 1,150,000 and 1,116,000 respectively on day 14. There were no significant differences in WBC or uric acid levels during the study. All red cell parameters returned to normal by day 35 in both ribavirin and ribavirin-GMP treated groups (I and III). All parameters in the control group (GMP) remained stable throughout the study.

There is no evidence that treatment with guanosine reduces the severity of ribavirin-induced anemia in rhesus monkeys. Anemia in monkeys treated with ribavirin-GMP was just as severe as that in monkeys given ribavirin alone. Treatment with GMP alone did not alter any parameters measured in this study and appeared to have no effect. In both the ribavirin-treated group and rivavirin-GMP treated group, recovery is rapid after termination of treatment, but is no more rapid in the ribavirin-GMP group than in the ribavirin group. Both groups display a similar pattern of anemia and thrombocytosis, followed by reticulocytosis and rapid recovery following ribavirin treatment.

Studies on Red Cell Deformability. Studies similar to those cited above have shown that rhesus monkeys develop an anemia during multiple, large dose treatments with ribavirin which suggests that ribavirin may affect either the osmotic fragility or deformability of erythrocyte. However, the results of a previous study (AVS-80-5) indicate that ribavirin does not significantly affect the osmotic fragility of RBCs. Hence, human and monkey RBCs were treated with ribavirin in vitro and tested for changes in deformability. To isolate RBCs heparinized blood was filtered through a 1:4 (v/v) mixture of microcrystalline cellulose and alpha cellulose, respectively, to remove most white cells and platelets, and resuspended 1:4 (v/v) in HBSS. Purified red cells were then incubated with 1 mg/ml of ribavirin for 2.5 hours at 37°C, then tested for deformability either immediately or after an additional period of incubation.

The RBC suspension was forced through a polycarbonate membrane under constant pressure and the pressure was monitored with a transducer indicator (%) and digital voltmeter and recorded by a strip chart recorder.

Tables II and III show that ribavirin does not appear to alter the deformability of erythrocytes as measured by the ability of ribavirin-treated cells to pass through a 3 pore filter as easily as untreated cells.

Internalization of VSV by Monkey Fibroblasts into Intermediate Vesicles. It has previously been shown using a series of two Percoll density gradients that human fibroblasts bind and internalize LDL (low-density lipoprotein), EGF (epidermal growth factor), and β-hexosaminidase (a lysosomal enzyme). These ligands are localized within 5 minutes in two structures of similar density.[4] These structures possess the endoplasmic reticulum marker, NADH-reductase, but lack enzymatic markers for lysosomes, Golgi and plasma membrane. Ligand contained in these intermediate structures is subsequently transferred to secondary lysosomes.

In order to obtain information of the route of internalization of VSV and on the site and possibly the mechanism of uncoating, the internalization of VSV into cultured monkey fibroblasts (Vero cells) was examined using a 2-step Percoll density gradient fractionation scheme. ^{35}S-labeled VSV was incubated with the cells at 4°C and also for various times at 37°C. Cells were then harvested, homogenized and the post-nuclear supernatants subjected to fractionation in 20% Percoll. The VSV was found to be distributed intracellularly (after 30 minutes at 37°C) in a single peak at a density of 1.040 g/ml. To examine the entrance of the VSV into intermediate vesicles, cells were incubated for 10 minutes at 37°C and fractionated in 20% Percoll. The peak of VSV-associated radioactivity was then pooled and subjected to further fractionation on a second Percoll gradient (9%).

These subcellular frationation studies have shown that the VSV, like LDL, EGF, and β-hexosaminidase are internalized by Vero cells and the VSV appears, within 5 minutes of incubation at 37°C, to be localized within two intermediate density structures distinct from plasma membrane and lysosomes. The virus becomes localized in secondary lysosomes within 20 minutes following incubation with cells at 37°C. In the presence of 16 mM NH_4Cl, VSV failed to enter secondary lysosomes and remained "trapped" in the intermediate structures.

These data show that the internalization of VSV is mediated through intermediate (non-lysosomal) vesicles in a manner similar to other physiological ligands. Currently, we are utilizing ^{32}P-labeled VSV in similar studies in order to examine when and where (cytoplasmically) the process of uncoating occurs. Electron microscopy and plaque assays will also be performed on the gradient fractions to understand the morphology of this process and to determine the infectivity of the internalized virus. Future plans include studies on the effect of pH and various antivirals, and lysosomotropic (such as weak bases) agents on the process of internalization and uncoating in several other virus models. Future experiments using this approach will also involve examining whether Diptheria toxin is or may be internalized through those intermediate structures and if the toxicity of this agent is related to these structures.

Role of Lysosomal Enzymes in Viral Uncoating. The proteolytic, lysosomal enzyme Cathepsin B, has been purified from calf liver. The purified enzyme and a variey of other proteolytic enzymes were incubated with ^{35}S-labeled, partially purified G glycoprotein (octyl-glucoside extracts of labeled virus) of VSV (provided by Dr. Douglas Miller, Rutgers Univ. Med. School). The enzyme was activated with 3 mM cysteine and the reaction carried out at 37°C for 15 min at pH 5.2. The reaction was stopped (with a solubilization solution containing SDS, β-mercaptoethanol, bromophenol blue, glycerol, Tris-HCl-buffer) and the proteolytic products analyzed by SDS-PAGE on 10% gels followed by autoradiography.

Preliminary results have shown that when compared to a variety of proteolytic enzymes, only Cathepsin B (pH optimum = 5.0 - 6.8) and Cathepsin D (pH optimum = 3.4 - 4.3) were capable of producing the cleavage in the G glycoprotein "spike" of VSV virus which is necessary to release Gs material. In infected cells the production and concomitant release of Gs from G to the media is thought to be related to the process of infection. It is thought that lysosomes and/or Cathepsin B and its endogenous inhibitor will be implicated as playing a role in the uncoating and cellular infection VSV and possibly other viruses.

Many cells and tissues have been shown to possess a naturally occurring inhibitor to Cathepsin B, a major intracellular protease, which in normal and aberrant physiological conditions is part of the cascade of enzymes known to control normal protein turnover as well as tissue atrophy and destruction. The inhibitor is being purified to homogeneity by: 1) homogenization; 2) heating (80°C) at pH 2.75; 3) 40-70% ammonium sulfate fractionation (at pH 6.5); 4) DEAE-sephagel chromatography (at pH 7.5); 5) gel filtration chromatography (at pH 6.0); and 6) isoelectric focusing. Antibodies will be raised to the purifid inhibitor so that the dynamic equilibrium maintained between the activity of the inhibitor and of Cathepsin B can be quantitatively monitored in cases of infection and disease. If lysosomal proteases (specifically Cathepsin B) and their endogenous inhibitors are involved in the process of viral uncoating and infection, then it is hoped that this may provide a broad point of attack for viral infections.

Mechanism of Action of Didemnins. Didemnins are a class of depsipetides recently isolated from a Caribbean tunicate of the Didemnidae family, a species of the genus Trididemnum. Didemnins have been shown to inhibit the growth of both RNA and DNA viruses in cell culture and in vivo. In cell culture, didemnins exhibit antiviral activity against Coxsackie, equine rhinovirus, herpes simplex type I and II, vaccinia, influenza, parainfluenza-3, RVF, SFS, PIC, and VEE viruses. The antiviral activity exhibited by the didemnins appears to be greater than any underlying cytotoxicity produced by the drug. In vivo, didemnins have been shown to protect mice against RVF and vaginal herpes simplex type II.

The mechanism of action of didemnins, however, is not known. Some preliminary data suggest that the drug may inhibit viral-induced protein synthesis. If the antiviral activity of didemnin is due to inhibition of viral-induced protein synthesis, the drug may play an important role in combination chemotherapy. In combination with drugs which inhibit viral-induced nucleic acid synthesis, didemnins may enhance the efficacy of known antiviral drugs.

To determine that the antiviral activity of didemnin is not due to cytotoxicity, the effect of didemnin A on macromoleuclar synthesis in Vero cells was investigated. Didemnin A was found to inhibit ^{3}H-leucine incorporation into macromolecules with an ID_{50} = 2.2 ± 0.5 µg/ml (N = 5). At the highest concentration of didemnin A used, 10 µg/ml, protein synthesis was inhibited 83.9 ± 4.6 (n=4)%. At this concentration of drug, cellular uptake of ^{3}H-leucine was inhibited 40.4%.

Preliminary data suggest that didemnin A, also inhibits ^{3}H-uridine incorporation into cellular macromolecules. The drug inhibits RNA synthesis by approximately 15%. Maximum inhibition occurs at 2.0 g/ml of didemnin A and remains constant up to 10.0 g/ml of drug. Didemnin A appears to have no effect on cellular uptake of ^{3}H-uridine.

The Mechanism of Binding of a Virus to Its Cell Surface Receptor and Subsequent Internalization. To follow-up on the observation that certain lectins especially Triticum vulgaris agglutinin (WGA), Glycine max agglutinin (SBA), and Canavalia ensiformis agglutinin (ConA) when prebound to cells, blocks TC-83 binding. The binding was further characterized by binding fluorescent labeled lectins to BW-J-M cells. All five lectins Arachis hypogaea agglutinin (PNA), SBA, Ricinus communis agglutinin I (RCA-I), WGA, and Ulex europaeus agglutinin I (UAE-I), fluorescein isothiocyanate labeled were incubated with cells at 0.01 and 0.1 µg/ml at 0°C for 60 min, and processed as described by Huggins et al.[5] When viewed with incident illumination and FITC selective filters, all lectins bound uniformly and essentially equally to the cells, suggesting that quantitative differences in lectin binding could not account for the differential inhibition of virus binding.

Most lectins bind to multiple glycoproteins on the cell surface, although notable exceptions of selective binding to a single cell surface glycoprotein have been reported by Huggins et al.[5] To determine how many glycoproteins the lectin was binding to, the lectin overlay technique of Burridge was used exactly as described by Huggins et al.[6] with ^{125}I labeled, PNA, ConA, DBA, SBA, RCA-I, WGA, and UEA-I. All seven lectins bound to multiple glycoproteins (7 to 20). By comparison of overlays from lectins that inhibited and failed to inhibit binding it appears possible to utilize lectin chromatography to at least partially purify the receptor.

To establish that TC-83, bound to BW-J-M under conditions used for the binding assay, could lead to productive infection, growth studies were undertaken. A growth curve was performed by infecting BW-J-M growing in monolayer with TC-83 (MOI = 10) and assaying virus in the supernatant by plaque assay. The virus yield and time course was much slower than expected for a fibroblast, raising the question if all cells were infected. To determine the percentage of cells infected, the growth curve was repeated and both the virus yield and percent infected cells were assessed. All virus inoculated BW-J-M cells, were positive by FA at days 1 to 5; however, fluorescence was significantly lower (+1) than in Vero cells which were infected and processed in parallel for comparison. Virus titer in BW-J-M cells rose 3 logs in two days; however, all cells remained viable up to day five, the last day tested. To establish conclusively that BW-J-M cells, infected by binding TC-83

under conditions used for the binding assay, were producing virus, an infectious center assay was used. The data assayed by probit showed each cell to be productively infected. From this data we conclude that the growth of TC-83 in BW-J-M cells is significantly different than in fibroblasts.

The binding of TC-83 to [illegible]lls was studied morpholoically by transmission electron microscopy. The vir[illegible]nown to bind preferentially to coated regions of the membrane corresponding to coated pits, an observation strongly supporting the theory that internalization occurs via endocytosis. No preferential binding to microvilli was seen.

From these studies the conditions necessary to reproducibly bind TC-83 to its high affinity receptor (which is shown to lead to productive infection) are established. To proceed to characterize the internalization, additional information of the process is required. To determine the time course of virus internalization it is necessary to have a measure of internalized virus.

To follow the pathway of virus internalization, identification of subcellular organelles that the virus is associated with at any given time is an important first step. This information can potentially be obtained biochemically by the co-localization of virus with biochemical markers of subcellular organelles (i.e., marker enzymes). To do this, the approach for separation of subcellular organelles fraction on density gradients was used. The method adapted by Canonico et al.[7] for primary mouse macrophages was used as a starting point. The method consists of disrupting cells by homoenization followed by a low speed centrifugation to remove nuclei and unbroken cells. The resulting fraction containing all the subcellular organelles except nuclei, was placed on a density gradient and subcellular organelles separated by their buoyant density. Fractionation of homogenates using a 25 to 55% w/v sucrose gradient in a VTi-50 vertical reorienting rotor showed separation into 4 subcellular fractions.

From initial studies tentative assignments for marker enzymes were made as follows: plasma membrane (WGA, 5' nucleotidase) Golgi(galactosyltransferase), mitochondria (malate dehydrogenase), lysosomes (n-acetyl-β-glucosaminidase), and endoplasmic reticulum (sulphatase C). Using the sucrose density gradients intracellular oranelles could be separated, however, when TC-83 was run in this system only marginal separation from plasma membrane was observed.

Thus, the alternative density gradient medium Percoll was adopted. Percoll is a self-forming density gradient medium. A gradient was designed that separated TC-83 from plasma membranes and from TC-83 bound to cells at 0°C. The final gradient utilizes Percoll-0.25 M sucrose and 1 mM EDTA (pH 6.8). The gradient separates subcellular organelles, although not as clearly as sucrose. Using this sytem, ^{35}S-TC-83 are warmed up to 37°C for 15 min, then washed and homogenized, 45% of the radioactivity was shifted to a denser portion of the gradient.

The shift was found to be difficult to reproduce and of a magnitude much lower than suggested by related experiments on the internalization of SFV in fibroblasts and epithelial cells. To resolve this, the binding of TC-83 to Vero-76 cells was characterized. A binding assay was developed in 6-well plates. ^{35}S methionine labeled TC-83 binds to Vero cells in a specific competitive manner, that is ^{35}S labeled virus binding can be reduced by competition with unlabeled virus. Preliminary data suggest a binding constant of the same order as TC-83 binding to BW-J-M cells. Binding consists of two components; a high affinity component and a

component of much lower affinity probably due to nonspecific binding. Virus bound at 4^0C did not come off the cell when cells were washed and reincubated at 4^0C. When cells were incubated at 37^oC, 20% of the virus came off during a 90 min period, an amount equal to the nonspecificity level. Virus was also presumably internalized and degraded, because TCA soluble material appears starting at 15 min. This data suggests that TC-83 in Vero-76 cells is processed in a manner similar to SFV. Studies are now underway to compare internalization of TC-83 in BW-J-M a macrophage-like cell line and Vero-76 cells.

Another aspect of this research unit concerns the basic mechanisms that regulate the coated vesicle pathways within cells. Calmodulin, a ubiquitous intracellular calcium regulatory protein, has been implicated in the function of coated vesicles. Current studies have been directed towards defining which of the constituent proteins of coated vesicles is the site of the calmodulin-coated vesicle interaction. Through the use of a photoaffinity derivative of radiolabeled calmodulin, it has been possible to identify three coated vesicle proteins that bind calmodulin. Exposure of purified coated vesicles to azido-^{125}I-calmodulin resulted in covalent labeling of three protein complexes with apparent molecular weights of 130,000, 93,000, and 52,000. The specificity of this labeling was demonstrated by its dependence on calcium, and by its reduction in the presence of unlabeled calmodulin or stelazine. Subtracting a molecular weight of calmodulin of 20,000 form the apparent weights of the covalently labeled complexes, the coated vesicle proteins that bind calmodulin are 110,000, 73,000, and 32,000 MW. The 32,000 MW protein is thought to participate in coat structure, but the other two are associated with the vesicle membrane and most likely participate in the interaction of clathrin the principal coat protein, with membrane to form a coated pit or coated vesicle. Known inhibitors of calmodulin will be tested in a virus-cell system to determine if the calmodulin inhibitors can disrupt virus internalization by the coated vesicle pathway.

The research described here should enable us to better understand: 1) the mechanisms by which viruses infect cells, and 2) the mechanisms by which coated pits and coated vesicles transport viruses, growth factors and certain toxic macromolecules.

Presentations:

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TABLE I. IN VITRO EVALUATION OF RIBAVIRIN ANALOGUES AND OTHER POTENTIAL ANTIVIRAL DRUGS AGAINST VARIOUS VIRUSES

WRAIR NUMBER	CONTRACT NUMBER	AVS #	RESPONSE OF VIRUS RVF[b]	VEE	PIC	YF	SF
	Didemnin "A",	160	0.0015	0.0005	0.003	0.0004	0.0004
	Didemnin "B",	161	$4x10^{-5}$	$7x10^{-5}$	$20x10^{-5}$	$7x10^{-5}$	$7x10^{-5}$
BK02664[a]	RA-0209	126	-	-	-	-	-
BK02673[a]	RA-210	125	-	-	-	-	-
BK02682	RA-211	128	-	-	-	-	-
BK02691[a]	RA-212	127	-	-	-	-	-
BK02708[a]	RA-213	124	-	-	-	-	-
BK02619[a]	RV-198	133	-	-	-	-	-
BK02628	RV-199	130	-	-	-	-	-
BK02637[a]	RV-210	272	-	1.04	.10	.55	-
BK02655[a]	RV-203	134	-	-	-	-	-
BK02600[a]	RY-91	129	3.4	-	-	-	-
BK02593[a]	RY-99	132	1.2	-	-	-	-
BK12633[a]	RA-20	73	.12	.32	.08	.03	.14
BK12526	RA-42	135	-	-	-	-	-
BK12571	RA-214	140	.03	.37	.19	.22	.22
BK1264[a]	RA-215	146	-	-	-	-	-
BK12535[a]	RA-216	136	.007	-	.55	1.33	-
BK12580[a]	RE-407	141	-	-	-	-	-
BK12599[a]	RE-410	209	-	-	-	-	-
BK12544[a]	RV-222	137	-	-	-	1.52	-
BK12553[a]	RV-223	138	-	-	-	-	.94
BK12562	RY-24	139	.048	.267	-	.011	-
BK12685	RY-48	144	.079	-	-	.079	-
BK12606[a]	RY-107	143	22	-	.154	.139	-
	NSC 115560	None	-	-	-	-	-
	NSC 45153-M	158	-	-	-	-	-
BK15661	RE-413	181	-	-	-	-	-
BK15670	RP-101 (II)	183	.033	-	-	-	-
BK15769	SB-13	185	.005	-	-	.111	.792
BK15723	RV-191	187	.001	-	-	-	-
BK15689	RP-150	189	.010	-	-	-	-
BK15741	RV-231B	191	-	1.31	-	.32	-
BK15714	RP-165 (II)	193	-	-	-	1.01	-
BK15732	RV-231-A	195	.002	1.31	-	.119	-
BK15778	SB-14	197	.003	2.62	-	-	2.62
BK15705	RP-165-I	199	.345	-	-	-	-
BK15796	SB-21	200	.006	-	.003	-	-
BK15787	SB-19	202	.008	-	2.03	1.38	-
BK15698	RP-160	204	.33	-	.50	.24	-

TABLE I. (continued)

WRAIR NUMBER	CONTRACT NUMBER	AVS #	RESPONSE OF VIRUS RVF[b]	VEE	PIC	YF	SF
BK15750	RV-247	206	.008	2.62	1.40	.40	.25
BK15652	RE-410	142	-	-	-	-	-
	Sodium Suramin	212	$70x10^{-5}$	.33	.066	.016	.213
	A-140	203	-	-	-	-	-
BK17245	RA-233	213	-	-	-	-	-
BK17227	RA-229	214	2.62	-	-	-	-
BK17405	RV-265	215	.039	-	.02	.11	.02
BK17343	RV-254	216	-	-	-	.40	-
BK17236	RP-232	217	2.37	-	-	-	.08
BK17307	RP-165 (I)	218	-	-	-	.76	-
BK17325	RP-170	219	.34	-	-	-	-
BK17254	RE-440	220	-	-	-	-	-
BK17263	RE-443	221	-	-	-	.47	-
BK17316	RP-165 (II)	222	-	-	.081	$43x10^{-5}$	-
BK17272	RE-445	223	-	-	-	-	-
BK17352	RP-173	224	-	-	.08	.40	-
BK17281	RE-454	225	2.03	-	-	-	-
BK17361	RV-253	226	-	-	-	-	-
BK17398	RV-264 (II)	227	-	-	.567	-	-
BK17389	VR-264 (I)	228	.003	-	.152	.820	.820
BK17290	RO-90 (RKR)	229	.04	-	.152	.125	-
BK17334	RP-171	230	-	-	.192	.02	-
	NSC 344209[c]	235	.91	-	.10	.04	.24
BK22657	RE-461	240	.085	-	-	0.85	-
BK22666	RE-465[a]	241	-	-	-	-	-
BK22728	SB-22	242	-	-	-	-	-
BK22648	RE-452	243	2.21	-	-	0.27	-
BK22684	RP-190	244	.007	.769	-	0.53	-
BK22693	RP-191	245	-	-	-	-	2.79
BK22746	SB-66	246	0.66	-	.002	.01	0.02
BK22737	SB-59B	247	.89	-	.04	-	-
None	RO-115	253	.005	$49x10^{-5}$	.005	$5x10^{-5}$	6.5
BK22719	RV-278	248	-	-	-	0.2	0.40
BK22639	RA-230	249	0.35	-	0.13	-	-.33
BK22700	RV-86	251	-	-	-	-	-
	Formycin B[d]						
	RA-138	65	1.87	-	0.37	-	1.43
	Ribavirin		1.09	2.46	.250	.190	.316

[a]Inhibitory dose 50 (μM).
[b]Indicates compounds tested in vivo also.
[c]Formycin B, Lot 800258.
[d]Sigma Chem. Co.

TABLE II. PERCENT RBC'S TRAPPED - HUMAN[a]

Incubated 2.5 hours		Incubated 24 hours	
Control, %	Treated, %	Control, %	Treated, %
0.37	0.38	0.14	0.16
0.29	0.32	0.17	0.18
0.37	0.41	0.25	0.23
0.37	0.39	0.26	0.26
mean = 0.35	mean = 0.37	mean = 0.25	mean = 0.21
s.d. = 0.04	s.d. = 0.04	s.d. = 0.06	s.d. = 0.05

[a]RBC concentration = 100 x 10^6 RBC/ml.

TABLE III. PERCENT RBC'S TRAPPED - MONKEY[a]

Incubated 2.5 hours		Incubated 24 hours	
Control, %	Treated, %	Control, %	Treated, %
0.14	0.14	0.13	0.09
0.13	0.13	0.09	0.10
0.12	0.13	0.13	0.11
0.12	0.12	0.11	0.09
mean = 0.13	mean = 0.13	mean = 0.11	mean = 0.10
s.d. = 0.00	s.d. = 0.01	s.d. = 0.02	s.d. = 0.01

[a]RBC concentration = 300 x 10^6 RBC/ml.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DAOG 3874	82 10 01	DD-DR&E(AR) 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
81 10 01	D. CHANGE	U	U		NL	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	131
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE *(Precede with Security Classification Code)*

(U) Role of Macrophage Proliferation and Activation in the Control of Viral Infections

12. SUBJECT AREAS

003500 Clinical Medicine; 004900 Defense; 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 05	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATE EFFECTIVE | EXPIRATION

b. CONTRACT/GRANT NUMBER

c. TYPE | d. AMOUNT

e. KIND OF AWARD | f. CUM/TOTAL

18. RESOURCES ESTIMATE

FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
82	2.6	121
83	3	127

19. RESPONSIBLE DOD ORGANIZATION

a. NAME: USA Medical Research Institute of Infectious Diseases

b. ADDRESS *(Include zip code)*: Fort Detrick MD 21701-5011

c. NAME OF RESPONSIBLE INDIVIDUAL: Barquist, R F

d. TELEPHONE NUMBER *(Include area code)*: 301-663-2833

20. PERFORMING ORGANIZATION

a. NAME: Bacteriology Division, USAMRIID

b. ADDRESS: Fort Detrick, MD 21701-5011

c. NAME OF PRINCIPAL INVESTIGATOR: Friedlander, A M

d. TELEPHONE NUMBER *(Include area code)*: 301-663-7341

21. GENERAL USE: FIC

MILITARY/CIVILIAN APPLICATION:

e. NAME OF ASSOCIATE INVESTIGATOR *(If available)*

f. NAME OF ASSOCIATE INVESTIGATOR *(If available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Viral Diseases; (U) Macrophages; (U) Cytotoxicity; (U) Immunity; (U) Laboratory Animals

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Study interaction of viruses and macrophages in order to be able to counteract viral diseases in military populations for which no therapy or immunization is possible. To stimulate appropriate macrophage proliferation in the soldier through the use of mediators such as the interferons. This should increase the ability of the soldier to function in a hostile, infectious disease environment.

24. (U) Develop experimental models of infection in experimental animals. Macrophages will be obtained from mice or guinea pigs of different ages, infected with model viruses to examine proliferative ability and examined for cytotoxicity.

25. (U) 8110 - 8209 - The proliferation of macrophages which is necessary for the expression of cell-mediated immunity can be inhibited in vitro with the arenavirus Pichinde. Another arenavirus, lymphocytic choriomeningitis virus has been found to act similarly. These experiments represent the first in vitro demonstration of interference with macrophage function by arenaviruses. Initial in vivo experiments confirm our in vitro data that Pichinde infection interferes with the macrophage's ability to proliferate in response to growth factors. These results may be related to the predilection of arenaviruses for the reticuloendothelial system and contribute to the immune suppression observed in these infections. The proliferating macrophage is not activated to possess enhanced antiviral activity as assessed by growth of viruses (Pichinde and Semliki Forest Virus) in macrophages or cytotoxicity of growth factor stimulated cells for viral infected targets. Thus, macrophage proliferation and the importance of macrophages in controlling many viral infections, either directly or indirectly through mediators such as interferon, attempts will be made to stimulate host macrophage production and activity.

BODY OF REPORT

Project No. 3A161101A91C: In-House Laboratory Independent Research (U)

Work Unit No. 91C LA 131: Role of Macrophage Proliferation and Activation in the Control of Viral Infections

Background:

The critical role of the macrophage in natural and acquired resistance to viruses is well recognized (1). This important role is supported by observations that the genetic and age-dependent resistance or sensitivity of animals, as well as the relative virulence of viral strains such as the arenavirus, lymphocytic choriomeningitis (LCM) (2), correlates with the ability of macrophages to replicate viruses in vitro. Furthermore, agents which stimulate or impair macrophage function can enhance or decrease resistance to experimental virus infections, e.g., Semliki Forest Virus (SFV) (3, 4). In addition, some agents, such as the arenaviruses, display a tropism for the host's macrophages (5, 6).

The macrophage as part of the reticuloendothelial system is often the first cell encountered by a virus. The early monocyte-macrophage inflammatory response may be an important determinant of resistance. Thus, the macrophage appears to be the prime effector cell in the non-specific phase of host resistance. During the development of cell-mediated immunity to intracellular pathogens, the macrophage, under the influence of the T lymphocyte, is stimulated to proliferate and to become activated (i.e., possess enhanced microbiocidal activity) as shown in the classic experiments of Mackaness. Thus during the course of a virus infection, there are more macrophages available to resist the virus. Whether the individual metabolically activated macrophage is better able to restrict the infection has not until now been determined. It is clear, though, that the macrophage is one of the important effector cells in cell-mediated immunity to viruses. Understanding the interaction between virus and macrophage, the virulence mechanisms of the virus which impair resistance, and the determinants for control of infection by the macrophage will eventually enable us to modulate macrophage function with natural or synthetic products to enhance resistance to infections in man.

Progress:

Effect of Pichinde infection on macrophage proliferation and function. Our previous studies have demonstrated that the arenavirus Pichinde (PIC), which shows a tropism for the reticuloendothelial system, causes a profound inhibition of peritoneal macrophage proliferation. These studies also demonstrated that the inhibition of macrophage DNA synthesis by PIC infection is not due to the production of soluble mediators such as prostaglandins or interferon (IF). However, we did find that PIC infection induced the production of IF by macrophages. In two experiments after 4 and 6 days of infection, IF could be detected in the medium from PIC-infected (but not control) macrophages at a titer of 10 units/ml. We also examined whether prior stimulation or macrophages by macrophage growth factor (MGF) would increase the production of IF after viral infection, because MGF has been reported to increase macrophage IF production after poly I:C (7). No such enhancement of IF production by MGF was seen after infection with PIC or SFV. Thus, MGF-stimulated macrophages do not have an enhanced ability to produce IF in response

to these viruses. However, neither of these viruses induce appreciable quantities of IF by macrophages and it will be of interest to study other viruses which are good IF inducers to determine whether MGF enhances IF production.

We also extended our prior studies which showed that PIC in doses which inhibited >75% of macrophage DNA synthesis had little if any cytopathic effect by examining Fc receptor activity of infected macrophages. As shown in Table I, PIC infection of macrophages had essentially no effect on the attachment of IgG-coated sheep RBC using both excess and limiting amounts of IgG. This agrees with reports that infection of macrophages with LCM or Junin viruses had no effect on phagocytosis (8, 9).

TABLE I. EFFECT OF PICHINDE INFECTION ON Fc MEDIATED PHAGOCYTOSIS

PARTICLE	% CELLS WITH ATTACHED E	
	UNINFECTED	INFECTED
E alone	2	2
EA (1:1000)	100	98
EA (1:2000)	91	91
EA (1:4000)	60	69
EA (1:8000)	21	22

Three days after infection with PIC, mouse peritoneal macrophages were tested for uptake of sheep RBC alone (E) or opsonized with varying dilutions of IgG anti-RBC (EA).

Effect of Pichinde infection of other cell types. To determine whether the inhibition of macrophages by PIC was limited to cells from the peritoneal cavity, we developed a culture system to measure DNA synthesis in mouse bone marrow-derived macrophages. Marrow cells from mouse femurs were cultured with MGF. After 5 days in culture, non-adherent macrophage precursors were infected with PIC and then allowed to differentiate and proliferate. Six days later, DNA synthesis was measured by ^{3}H-thymidine incorporation. Prior infection with PIC at an MOI 5 caused a 45% inhibition of DNA synthesis as shown in Table II. This is significantly less inhibition than observed after infection of peritoneal macrophages at the same MOI (90-95%). Thus, macrophages from different anatomic sites and/or different stages of differentiation vary in their sensitivity to inhibition of growth by PIC infection. These studies obviously relate to the observation of Bro-Jorgenson and others (10) on the transient hematopoietic deficiency and decreased marrow colony-forming units associated with acute infection of the mouse with LCM. These authors were unable to show direct inhibition of marrow colony-forming units by LCM. Further studies with PIC in vivo will be of interest in this regard.

TABLE II. INHIBITION OF DNA SYNTHESIS IN BONE MARROW MACROPHAGE PRECURSORS BY PICHINDE INFECTION

	^{3}H-THYMIDINE INCORPORATION (CPM ± SEM)
Control uninfected	50,098 ± 3, 188
PIC (MOI = 0.1)	48,044 ± 3,383
PIC (MOI = 5)	27,565 ± 3,109

Experiments were also performed using starch-elicited peritoneal macrophages from suckling mice. It was found that suckling mouse macrophages also synthesize DNA in response to MGF. Furthermore, they appear similarly sensitive to inhibition by PIC infection. This suggests that the differential sensitivity of the suckling mouse to PIC infection in vivo is not due to a differing ability to inhibit macrophage DNA synthesis. Further studies of viral growth are necessary to confirm the suggestion that the macrophage is not responsible for the sensitivity of the suckling mouse to infection.

We also studied the effect of PIC on DNA synthesis in another cell type, the fibroblast. Using mouse fibroblasts derived from muscle, it was found that PIC infection did not inhibit DNA synthesis as it did in mouse macrophages. This most likely reflects the fact that macrophages are more permissive than fibroblasts for PIC (only 10-20% of fibroblasts compared to 90% of macrophages were positive for PIC by immunofluorescence). This may help explain the predilection of PIC for cells of the reticuloendothelial system.

Effect of other viruses on macrophage proliferation. Experiments with other classes of viruses included VEE (TC83 and Trinidad), West Nile, Banzi, SFV and Rift Valley Fever (RVF). Only RVF among these viruses caused > 50% inhibition of MGF induced DNA synthesis. This agrees with studies of Rosebrock and Peters showing that RVF is toxic for rat macrophages.

Of the arenaviruses examined so far, LCM but not Junin virus (XJ44 and Romero), caused significant inhibition of macrophage DNA synthesis (Table III). This may reflect the differential permissiveness of macrophages for these viruses (8, 9).

Mouse peritoneal macrophages were cultured at 1.5 X 10^5 cells/cover slip and infected at various MOI. Two days later, MGF was added along with ^{3}H-thymidine. DNA synthesis was measured 4 days (incorporation was low in the Junin experiment because of the unfavorable cultural conditions in Dr. Kenyon's Laboratory).

TABLE III. INHIBITION OF MACROPHAGE DNA SYNTHESIS BY LCM AND JUNIN VIRUSES

	^{3}H-THYMIDINE INCORPORATED (CPM ± SEM)
Experiment 1	
Control Uninfected	23,986 ± 956
LCM (WE) (MOI =.005)	4,159 ± 181
LCM (WE) (MOI = .05)	4,089 ± 596
LCM (WE) (MOI = 0.5)	7,948 ± 471
Experiment 2	
Control Uninfected	3,869 ± 395
XJ44 (MOI = .01)	4,159 ± 134
XJ44 (MOI = 1)	6,774 ± 523
Romero (MOI = .01)	5,966 ± 979
Romero (MOI = 1)	4,480 ± 754

Effect of acute Pichinde infection on macrophage function in mice. We have begun initial studies of PIC infection in vivo in mice. These have revealed that intraperitoneal inoculation of PIC causes a significant decrease in the number of macrophages recovered from a peritoneal exudate. The effect is most evident when virus is introduced on the same day as the starch used to elicit the exudate, that is, 4 days before the cells are harvested. Inactivated virus had no such effect. Of equal significance is that the macrophages recovered were unable to synthesis DNA in response to MGF(Table IV). Thus, in vivo infection with PIC reproduces the observations made in vitro. We plan to examine this in more detail as the early mononuclear exudate response may be a critical factor in resistance to infection.

Mice were inoculated IP with mock medium, cobalt inactivated or live PIC, and later in the day with 2% starch. Four days later peritoneal macrophages were harvested and cultured. Two days later, MGF and ^{3}H-thymidine were added and DNA synthesis measured four days later. Data represent the mean ± SEM of five mice per group.

TABLE IV. EFFECT OF PICHINDE INFECTION IN VIVO ON THE ABILITY OF MACROPHAGES TO SYNTHESIZE DNA

SOURCE OF MACROPHAGES	^{3}H-THYMIDINE INCORPORATED (CPM ± SEM)
Mock infected medium	11,966 ± 1,004
Cobalt inactivated PIC	14,398 ± 534
PIC	1,043 ± 235

Macrophage antiviral activity: cytotoxicity of macrophage for viral infected cells. The P815 mouse mastocytoma cell line has been successfully infected with SFV and used as a target cell in a ^{51}Cr assay for macrophage cytotoxicity. P815 cells are infected with SFV for 7 h and then labeled with ^{51}Cr for 1 h, washed, and added to cultures of macrophages. Specific ^{51}Cr release is determined 16 h later. In this system, normal macrophages were not cytotoxic for either uninfected or SFV infected P815 cells. Thus normal exudate macrophages are not cytocidal for viral-infected targets. Exposure of macrophages to MGF for periods up to 3 days did not stimulate macrophages to become turmoricidal nor to lyse viral-infected target cells. These experiments show that MGF stimulated macrophages are not cytotoxic for viral infected cells.

We prepared lymphokines using mitogen-stimulated spleen cells from syngeneic animals and studied their effect on macrophage antiviral activity. These lymphokines (in the presence of endotoxin) could induce macrophages to become cytotoxic for uninfected P815 tumor cells as reported by other workers showing that these macrophages could be activated. However, the lymphokines did not enhance the cytotoxicity of macrophages for viral infectd targets. In addition, MGF-treated macrophages did not show an increased cytotoxic response to the lymphokines. Thus, lymphokines do not enhance the antiviral activity of macrophages as measured by cytotoxicity. Experiments to determine the effect of lymphokines on viral growth in macrophages are in progress.

Growth of SFV in macrophages. To determine further the relationship between macrophage proliferation and antiviral activity, we studied the effect of MGF on the replication of SFV in macrophages. As with our prior studies with PIG, MGF had no effect on the replication of SFV in macrophages (Table V). Similar results were observed with mouse virulent and avirulent strains of SFV. Thus, using two unrelated viruses, the results show that MGF-stimulated macrophages do not have an enhanced ability to limit virus replication.

TABLE V. EFFECT OF MGF ON THE GROWTH OF SEMLIKI FOREST VIRUS IN MACROPHAGES

DAY OF INFECTION	CONTROL MACROPHAGES	MGF TESTED MACROPHAGES
0	1.63×10^{2}*	1.13×10^{2}
1	1.28×10^{6}	6.90×10^{5}
2	3.43×10^{5}	1.83×10^{5}
3	7.13×10^{4}	4.10×10^{4}
4	2.73×10^{4}	8.47×10^{3}

*PFU/ml

Mouse peritoneal macrophages were cultured in control or MGF containing medium for 4 days and then infected with SFV. Viral growth was measured daily.

Presentations:

1. Friedlander, A. M. Inhibition of macrophage DNA synthesis by infection with the arenavirus Pichinde. Presented, International Reticulo-endothelial Society Meeting, Davos, Switzerland, February 1982.

2. Friedlander, A. M. and P. Jahrling. Effect of macrophage growth factor on antiviral activity of mouse peritoneal macrophages. Presented, Federation of American Society of Experimental Biology, New Orleans, LA, April 1982.

Publication:

1. Friedlander, A. M., P. B. Jahrling, P. Merrill, and S. Tobery. 1984. Inhibition of mouse peritoneal macrophage DNA synthesis by infection with the arenavirus Pichinde. Infect. Immun. 43:283-288.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DAOG 3875	82 10 01	DD-DR&E(AR) 636
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISS'N INSTR'N	9. LEVEL OF SUM / A. WORK UNIT
81 10 01	D. CHANGE	U	U		NL	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	132
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE *(Precede with Security Classification Code)* (U) Role of T-Cells in Pathogenesis of Argentine Hemorrhagic Fever

12. SUBJECT AREAS
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 06	83 06	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	2.4	182
c. TYPE	d. AMOUNT	83	2.0	155
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS *(Include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR Kenyon, R H
d. TELEPHONE NUMBER *(Include area code)* 301-663-?833	d. TELEPHONE NUMBER *(Include area code)* 301-663-7241
21. GENERAL USE FIC MILITARY/CIVILIAN APPLICATION:	f. NAME OF ASSOCIATE INVESTIGATOR *(If available)* g. NAME OF ASSOCIATE INVESTIGATOR *(If available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) BW Defense; (U) Military Medicine; (U) Lab Animals; (U) Argentine Hemorrhagic Fever; (U) Arenaviruses; (U) Immunology

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Determine pathogenesis of Argentine hemorrhagic fever (AHF) in animal models. Application of these principles to testing the effect of immune serum, antiviral drugs and experimental vaccines on the course of disease will be conducted. Such information will be of direct value in developing medical defenses for U.S. Military populations that may be exposed to this and other arenavirus infections.

24. (U) After determining the effector mechanism for cytotoxicity in a tissue culture system, attempt to correlate such a mechanism in a guinea pig model with protection or disease induction. Efforts then will be made to manipulate the mechanism(s) for the benefit of an infected individual.

25. (U) 8106 - 8209 - A cytotoxicity assay for the causative organism of AHF (Junin virus)-infected cells by spleen cells from convalescent guinea pigs has been developed and is believed to be due to antibody-dependent cell-mediated cytoxicity (ADCC). A possible model for virulent Junin infection has been shown in cyclophosphamide-treated guinea pigs with attenuated virus. Results have shown that virulent Junin virus replicated well in vitro in mouse macrophages, while attenuated virus replicates poorly or not at all.

BODY OF REPORT

Project No. 3A161101A91C: In-House Laboratory Independent Research (U)

Work Unit No. 91C-00-132: Role of T-Cells in Pathogenesis of Argentine Hemorrhagic Fever

Background:

Argentine hemorrhagic Fever (AHF), an arenavirus infection caused by Junin (JUN) virus, is an acute severe disease with a mortality of about 15%. AHF is maintained in nature in rodent reservoirs, and is probably spread by aerosol routes. Other than supportive care, the only effective treatment of the disease is immunotherapy, which reduces mortality, but has been implicated with relapse presenting with neurological manifestations (1). This is similar to observations with immunoglobulin-treated Machupo-infected monkeys.

There are numerous analogies between JUN and lymphocytic choriomeningitis (LCM) viruses. Both are lymphotrophic, immunosuppressive, and cause hemopoietic dysfunction (2-4). Although little work along these lines has been done with JUN, studies have shown that much of the LCM pathogenesis in mice is due to cytotoxic T-cells (5). Due to the analogies of LCM and JUN viruses, the fact that neutralizing antibody cannot be measured until late in disease, and since the only accepted treatment leads to a significant rate of late neurological symptoms, the role of T-cells in pathogenesis of AHF warranted investigation.

Progress:

Studies have continued on cell-mediated cytotoxicity for AHF in inbred strain 13 guinea pigs. Using ^{51}Cr labeled target cells and guinea pig spleen cells as effectors to measure cytotoxicity, lytic units per spleen are shown in Table I. Cytotoxicity apparently peaks from days 10-15, and diminishes, but remains existent at day 30. Although ^{51}Cr release can be blocked by aggregated human IgG, suggesting antibody dependent cell-mediated cytotoxicity (ADCC), current attempts to duplicate the system using exogenous antibody and normal spleen cells have beer unsuccessful. Current efforts are concentrated in this area. Recent studies have indicated that guinea pigs infected with a virulent strain of AHF (Romero) also exhibit cytotoxic activity, similar to that shown earlier with a virulent strain (WE) of LCMV. Such Romero spleens also mediate classical ADCC against Vero cells (with added antibody). This suggests that early in the virulent infection antibody production is initiated and effector cells are competent. In spite of such immunocompetence at day 10, the Romero-infected guinea pigs proceed to die by days 15-17, perhaps due to progression of the disease resulting in severe immunosuppresssion after day 10.

Cyclosporin A is a drug which nearly selectively depletes T-killer cells in laboratory animals and in man. Experiments were performed to determine the effect of this drug on virulent and attenuated JUN infections of guinea pigs (Table II). Animals receiving virulent virus showed no change in death pattern in response to cyclosporin A and animals receiving attenuated virus and cyclosporin A showed 3/4 deaths. This suggests that if T-killer cells are the only population of T-cells

affected by the drug, then these cells may not play a detrimental role in pathogenesis but may play a role in controlling the virus infection.

Experiments were performed to determine whether a role for complement could be detected in AHF infections. Complement might function as an antiviral or antivirally infected cell mechanism or as an effector mechanism in host damage. The ability of fresh monkey serum to spontaneously inactivate JUN virus prepared in Vero cells is shown in Table III. Attenuated (Candidate I, XJ-44), virulent (Romero, Suarez) and semivirulent (Coronel) strains of JUN are inactivated in the presence of fresh serum, but not in heat inactivated serum. There is apparently no relationship between virulence and complement susceptibility. Appropriate studies indicated that the classical complement pathway is the mediator and that inactivation is not due to a naturally occurring anti-host cell antibody.

Experiments were performed to determine whether cells expressing viral cell surface antigens fixed complement or were lysed by complement. Lysis of infected cells by complement might serve the host in eliminating infected cells in vivo or could be detrimental by assisting in disseminating virus. Vero cells were infected with an MOI of 1 PFU to 20 cells, and trypsinized to remove cells and to plant on coverslips 24 hr prior to assay. Results of immune adherence as a measure of C-3 deposition on infected cell surfaces are presented in Table IV. Human red blood cells adhere via their C-3 receptor to cell surfaces where the complement cascade has been initiated (presumably by viral antigens) and C-3 deposited on the cell surface. Results showed a significantly greater number of rosetting cells in the infected cultures as compared with the control. Other results (not shown) suggested greater ^{51}Cr release from infected versus non-infected Vero target cells in response to complement. Studies on the role of complement in the pathogenesis of JUN in vivo are in progress.

Experiments were performed to determine whether the parent host cell plays a role in the virus susceptibility to complement. JUN Candidate I virus was grown in FRhL or Vero cells and progeny virus tested for inactivation in fresh monkey serum. Results (Table V) show that virus grown in FRhL cells is not inactivated in fresh serum in contrast to the inactivation seen with Vero cell-grown virus. Apparently, a host cell modification of virus membrane occurs which influences susceptibility to complement. Such a host cell modification could be important in the infection process in vivo.

Experiments were performed to determine the effect of ribavirin on JUN infections in guinea pigs. Guinea pigs were infected with a virulent strain of JUN and treated with 45 mg/kg/day drug for various periods of time as shown in Table VI. Ribavirin alone or monobutyrl ribavirin (MBR) alone for the time intervals we studied does not appear to have an effect on guinea pigs. Final outcome in infected-treated groups as measured by deaths/total is not affected by drug treatment, but a significant delay in time to death is noted. Since ribavirin most likely does not cross the blood brain barrier, it is not unexpected to find that those guinea pigs infected IP and treated with drug experience their highest organ titer in the brain and these groups exhibit the highest percentage of paralysis. In contrast, similarly infected animals with no drug treatment die sooner than the treated animals and no virus can be detected in the brain, but are found in other organs examined. Animals inoculated I.C. with JUN present a systemic disease pattern similar to IP inoculated animals. IC inoculated animals show virus throughout the organ systems including the brain. None of these IC-inoculated, nontreated animals has exhibited paralysis. When IC inoculated guinea pigs are

treated with ribavirin, the brain appears to be the most susceptible organ (as shown by organ virus titer) and most of these animals exhibit paralysis prior to death. Thus, neurovirulence of Junin for guinea pigs may be masked by their early death from hemorrhagic fever.

Table VII shows the effect of (MBR) on AHF infection in guinea pigs. MBR is thought to more easily cross the blood brain barrier. Results with MBR are essentially the same as those with ribavirin.

Immunotherapy is the recommended treatment for AHF and a significant percent of such treated individuals return with late neurological symptoms. We performed a study to determine the passive titer necessary to prevent death, effect of time after infection and frequency of therapy, and to experimentally produce a model of the late neurological disease. Results are summarized in Table VIII. Although amount and frequency of immunotherapy affect the outcome, results suggest treatment should not be delayed beyond day 3. Only 1 of 24 animals showed a true late neurological death (day 41). Other animals developed paralysis on day 11-21, and in nearly all these cases the highest virus titer was found in the brain. One possible suggestion is that with immunotherapy, antibody passes less freely to the brain than to other organs. Under conditions of limited antibody, this renders the brain susceptible to virus. For instance, when minimal treatment (0.6 ml) is administered on day 3, 9, 15, 21, 40% of the animals die with paralysis with the highest virus titer in the brain. Since few deaths have occurred in humans with late neurological syndrome, correlations of brain-associated virus with the syndrome are unknown. Despite the differences in time to paralysis, the same mechanisms may or may not function for this syndrome in both guinea pigs and humans.

Passive antibody titers (80% plaque reduction) were monitored in control groups of animals (no virus). Animals maintaining a 1:128 or greater titer for at least 21 days appeared to be protected from disease, while those with titers which dropped below this value in this time period eventually became ill and died.

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TABLE I. CYTOTOXIC ACTIVITY OF SPLEEN CELLS FROM AHF[a] INFECTED GUINEA PIGS

DAY P. I.	LYTIC UNITS/SPLEEN
3	N.O.[b]
6	2.1×10^6
8	3.6×10^7
10	4.3×10^7
13	4.0×10^7
15	3.1×10^7
30	4.5×10^6

[a] XJ44 Strain Junin
[b] N.O. = None observed

TABLE II. EFFECT OF CYCLOSPORIN A ON JUNIN INFECTIONS OF GUINEA PIGS

TREATMENT	VIRUS	NO. DEAD TOTAL	DAYS TO DEATH	VIRUS ISOLATION
Cyclosporin A[a] da 0 thru 14	None	0/6	-	-
None	Romero[b]	4/4	16 thru 18	N.D.
None	XJ-44[c]	0/4	-	None (one examined day 22)
Cyclosporin A da 0 thru 14	Romero	6/6	15 thru 18	N.D.
Cyclosporin A da 0 thru 14	XJ-44	3/4	22 thru 23	All organs tested serum -

[a] 25 mg/kg/day I.M.
[b] 5,000 PFU, I.P.
[c] 500,000 PFU, I.P

TABLE III. SPONTANEOUS INACTIVATION OF JUNIN VIRUS IN MONKEY SERUM USED AS A SOURCE OF COMPLEMENT

VIRUS STRAIN	MINUTES PFU/ml 0	30	60
Candidate I			
fresh	2.1×10^4	1.5×10^3	8.3×10^2
heat inactivated	2.4×10^4	4.5×10^4	2.3×10^4
XJ-44			
fresh	5.5×10^3	1.0×10^3	5.0×10^2
heat inactivated	3.0×10^4	2.5×10^4	2.3×10^4
Coronel			
fresh	2.2×10^4	2.8×10^3	2.3×10^3
heat inactivated	2.4×10^4	2.0×10^4	9.0×10^3
Romero			
fresh	2.3×10^3	2.0×10^2	8.8×10^1
heat inactivated	2.8×10^3	2.9×10^3	3.0×10^3
Suarex			
fresh	4.8×10^4	3.8×10^3	2.0×10^3
heat inactivated	5.0×10^4	4.8×10^4	4.7×10^4

TABLE IV. DEVELOPMENT OF JUNIN-SPECIFIC CELL SURFACE ANTIGENS AND THEIR REACTION WITH COMPLEMENT

DAY POST INFECTION	% CELLS POSITIVE FOR CELL SURFACE JUNIN ANTIGEN	% CELLS WITH POSITIVE ROSETTES	
		Infected	Uninfected
1	8	2%	2%
2	17	14%	6%
3	100	N.D.	N.D.
4	100	30	%5%

TABLE V. EFFECT OF HOST CELL ON SUSCEPTIBILITY OF JUNIN VIRUS FRESH MONKEY SERUM

	TIME (MIN,					
	FRESH SERUM			HEAT INACTIVATED SERUM		
	0	30	60	0	30	60
FRhL-Grown Candidate I	2.1×10^4	1.1×10^4	1.2×10^4	1.2×10^4	1.2×10^4	1.1×10^4
Vero Grown Candidate I	5.2×10^4	1.7×10^3	9.5×10^2	5.5×10^4	3.8×10^4	2.5×10^4

TABLE VI. EFFECT OF RIBAVIRIN TREATMENT ON AHF[a] INFECTION OF GUINEA PIGS

ROUTE OF INFECTION	TREATMENT DAYS	VIRUS ISOLATION	OUTCOME NO. DEAD/TOTAL	TIME TO DEATH (Days)	PARALYSIS NO./TOTAL
None	-1 thru 28	N.D.	0/12	-	0
None	-1 thru 14	N.D.	0/16	-	0
I.P.	-1 thru 14	1 Paralyzed; virus isolated from the brain	7/7	25,26	1.7
I.P.	+7 thru 24	[b] + all organs; highest titer brain	8/8	20 thru 30	3/8
I.P.	-1 thru 24	Highest titers brain; in both paralyzed and non-paralyzed	8/8	21 thru 28	4/8
I.P.	None	Highest titer lymph nodes and spleen; no virus in brain	12/12	14 thru 17	0/12
I.C.	-1 thru 14	All organs +; highest titers brain	5/5	16 thru 21	0/12
I.C.	None	all organs +; highest titers spleen and lymph nodes	5/5	13 thru 15	0/5

[a] 5,000 PFU Romero strain
[b] + = positive

TABLE VII. EFFECT OF MONOBUTYRLRIBAVIRIN ON AHF INFECTION IN GUINEA PIGS

ROUTE OF INFECTION	TREATMENT DAYS	OUTCOME NO. DEAD/TOTAL	TIME TO DEATH (Days)	PARALYSIS NO. TOTAL
I.P.	None	6/6	9 Thru 11	0/6
I.C.	None	4/4	12 thru 12	0/4
I.P.	-1 thru +14	6/6	15 thru 17	1/6
I.C.	-1 thru +14	4/5	12 thru 14	3/5
None	-1 thru 14	0/5		0/5

TABLE VIII. IMMUNOTHERAPY OF AHF-INFECTED GUINEA PIGS

VIRUS[a]	TREATMENT (ml antisera)	DAYS (P.I.) OF TREATMENT	DEATHS/ TOTAL	PARALYSIS NO/TOTAL
Romero, IP	12	0, 6	0/6	0/6
Romero, IP	6	0, 6	1/24	1/24[b]
Romero, IP	6	3, 9	0/6	0/6
Romero, IP	6	6, 12	6/6	0/6
Romero, IP	6	9, 18	6/6	0/6
Romero, IP	2	0, 6	7/11	0/11
Romero, IP	0.6	0, 6	9/11	0/11
Romero, IP	2	0, 6, 12, 18	0/4	0/4
Romero, IP	0.6	0, 6, 12, 18	1/10	1/10[b]
Romero, IP	2	3, 9, 15, 21	0/4	0/4
Romero, IP	0.6	3, 9, 15, 21	2/5	2/5[b]
Romero, IP	0.6	4, 10, 16, 22	4/5	1/5[b]
Pasadas, IP	6	0, 6	0/6	0/6
Romero, IP	None		20/20	0/20
Pasadas, IP	None		4/4	0/4
Romero, IC	2	0, 6	5/5	5/5[2]
Romero, IC	2	0, 6, 12, 18	2/5	2/5[2]
Romero, IC	None		10/10	

[a] 5,000 PFU
[b] Virus isolated from brain

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DAOG 3877	82 10 01	DD-DR&E(AR) 636
3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISS'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
81 10 01	D. CHANGE	U	U		NL	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	133
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE *(Precede with Security Classification Code)*
(U) Role of Anthrax Toxin Components in Virulence of *B. anthracis*

12. SUBJECT AREAS
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
81 06	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	2.4	317
c. TYPE	d. AMOUNT	83	1.5	100
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS *(Include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR Leppla, S H
d. TELEPHONE NUMBER *(Include area code)* 301-663-2833	d. TELEPHONE NUMBER *(Include area code)* 301-663-7241
21. GENERAL USE FIC MILITARY/CIVILIAN APPLICATION:	e. NAME OF ASSOCIATE INVESTIGATOR *(If available)* g. NAME OF ASSOCIATE INVESTIGATOR *(If available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) Military Medicine; (U) BW Defense; (U) Anthrax; (U) Protective Antigen; (U) Edema Factor; (U) Cell Culture

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Purify and characterize the three protein components of anthrax toxin, determining their physiological and enzymatic actions so as to allow rational design of measures to prevent and treat anthrax infections. Since anthrax is considered to be a prime BW candidate, it is essential to improve vaccines and treatment regimens for U.S. military forces who may be required to fight in a BW environment.

24. (U) Develop methods to produce and purify anthrax toxin; develop sensitive immunochemical, electrophoretic, and enzymatic assays to measure the toxin components; study the genetic mechanisms which control toxin synthesis.

25. (U) 8110 - 8209 - The three protein components of anthrax toxin were purified and characterized. Yields, purities, and subunit molecular weights were respectively, PA: 50 mg, >95%, 85,000 daltons; LF: 10 mg, >90%, 83,000 daltons; EF: 2 mg, >90%, 89,000 daltons. The heat stable eucaryotic substance required by EF to express its latent cyclase activity was shown to be calmodulin. Antisera were raised to each component and used in ELISA tests which, for PA, could detect as little as 1 ng toxin. LF (with PA) was shown to inhibit the growth of many types of cultured cells. However, one cell line (Chinese Hamster Ovary cell) grows in the presence of toxin and this discovery may lead to an unlocking of the enzymatic activity of LF. Lymphocyte hybridoma studies, concerned with producing monoclonal antibodies to anthrax toxins, are underway. Initial efforts are focused on antibodies to LF. To date, data suggest that EF toxin is not recognized by the experimental host as a foreign protein. A gene library of *B. anthracis* DNA was constructed in *E. coli*.

BODY OF REPORT

Project No. 3A161101A91C: In-House Laboratory Independent Research (U)

Work Unit No. 91C 00 133: Role of Anthrax Toxin Components in Virulence of *B. Anthracis*

Background:

The factors which make *Bacillus anthracis* virulent for man and animals are poorly understood. Rational design of preventive and therapeutic measures to control anthrax infections is therefore difficult. Previous work identified 3 proteins, collectively called "anthrax toxin," which contribute to pathogenesis. Of these 3 proteins, the protective antigen (PA) has been purified and used as a vaccine, but the lethal factor (LF) and edema factor (EF) have not been available in amounts adequate for characterization of their chemical structures or determination of their cellular modes of action.

This work unit has as its objective (a) purification of the protein components of the toxin, (b) development of immunochemical and physical assays to detect and quantitate the proteins, (c) discovery of animal models and tissue culture systems sensitive to the toxin, (d) identification of the fundamental subcellular processes affected by the toxin, and (e) discovery of the individual enzymatic reaction or critical cellular component altered by the toxin.

Progress:

Methods for the purification of the 3 anthrax toxin components that were described in the FY 81 annual report were further refined during this period. The Sterne strain of *B. anthracis* was grown in 20-L batches of R medium, a completely synthetic medium developed by investigators in the Bacteriology Division, USAMRIID. This medium, optimized for production of rat lethal toxin, appears to favor production of LF while maintaining levels of PA at least equal to those obtained in medium 1095. The production of EF in R medium compared to other media has not been measured. Using chromatography on DEAE and hydroxylapatite columns, the 20-L cultures yielded 50 mg PA, 10 mg LF, and 2 mg EF. Each of these materials was more than 90% pure when examined by SDS slab gel electrophoresis. The molecular weights determined from these gels were 85,000 for PA, 83,000 for LF, and 89,000 for EF. The LF purified in this way proved more toxic than earlier samples. Thus, 2 μg LF mixed with 100 μg PA killed Fisher 344 rats in 120 minutes. This LF is therefore 10-fold more potent than samples described in the FY 81 report.

Limited studies were performed to try to increase the production of EF. It was found that addition of horse serum to R medium or to brain heart infusion medium greatly enhanced EF recovery as measured by adenylate cyclase assay. Although originally thought to act by protection of the EF from proteolysis, the horse serum appears to have other actions since dialyzed serum is ineffective. Since large amounts of protein would complicate purification of the EF, attempts were made to replace the serum. However, no combination of protease inhibitors and nutrients has yet been found that approaches the effectiveness of horse serum. These experiments demonstrated that iron represses EF production, an effect seen with several other bacterial toxins.

Enzymatic studies continued during FY 82 to extend the fundamental discovery that EF is an adenylate cyclase. Initial work sought to identify the substance required for expression of the latent enzymatic activity of EF. Activation was achieved by a wide variety of materials, including fetal calf serum, casein, creatine phosphokinase, and boiled cell extracts. Enzymatic activity also was found to be susceptible to variations in the divalent ion content of the reaction mixture. Analysis of this fact led to the finding that activity was blocked by the calcium ion chelator, EGTA. It had previously been shown that an adenylate cyclase from Bordetella pertussis requires the heat stable, calcium binding protein calmodulin for activity (1). This protein is ubiquitous in eukaryotes, where it is an essential co-factor of many different enzymes, but it is not found in any prokaryotes. A sample of purified calmodulin was obtained from Dr. C. Linden, Physical Sciences Division, and was found to fully activate EF. Thus, it appears that EF has the unique property of requiring a normally intracellular eukaryotic protein for activity. This requirement is consistent with the model previously proposed which suggests that EF enters into the cytoplasm of target cells where it converts ATP to cAMP.

In many respects EF appears to resemble the previously isolated pertussis cyclase, except that EF appears to behave in a more uniform manner. Thus, the pertussis cyclase can be obtained in several forms of differing purity, each of which shows a different degree of dependence on calmodulin. In contrast, all samples of EF, including crude culture supernatants, show an absolute dependence on calmodulin, i.e., no adenylate cyclase activity can be detected in the absence of calmodulin. Another type of evidence showing the similarity of these cyclases comes from studies on lipid activation. The activity of the pertussis enzyme can be further enhanced when certain lipids are added in addition to calmodulin. Preliminary experiments indicate that a similar effect is found with EF.

Further characterization of the enzymatic properties of EF was performed using photoaffinity labeling techniques. EF was supplied to Dr. Boyd Haley, University of Wyoming, who demonstrated that EF becomes labeled when incubated with ^{32}P-azido-ATP and exposed to light. Labeling was strictly dependent on addition of calmodulin and calcium, and occurred in a protein of MW about 90,000. This is the first direct evidence that the major, high molecular weight protein in the purified EF samples is the enzymatically active species.

In their dependence on calmodulin, the pertussis and anthrax cyclases resemble adenylate cyclases isolated from certain animal tissues. The cyclase from cow brain is the best characterized of these calmodulin-dependent eukaryotic cyclases (2). The similarity in properties suggests the possibility that these two bacterial cyclases originated as eukaryotic cyclases, and that a gene for the eukaryotic cyclase was captured by the bacteria in a rare recombination event and maintained because the ability to make cyclase conferred a selective advantage. Eukaryotic cyclases are membrane-bound enzymes present in small numbers, and are therefore difficult to study. If a genetic or antigenic similarity between EF and eukaryotic cyclases could be demonstated, then EF could be a valuable tool in studying the eukaryotic cyclases. Two approaches are being used to measure the degree of similarity between EF and brain cyclase. One of these compares the enzymatic properties of the proteins. Although the brain cyclase has not been purified, some of its enzymatic properties are known. One characteristic feature is that 1 mM calcium ion inhibits the cyclase activity when the major divalent ion is magnesium, but not when it is manganese. When the calcium response of EF was examined, it was found to match that described for brain cyclase.

The second test of the similarity of EF to brain cyclase employs specific antisera raised against EF. Specific antisera to PA and LF were obtained without difficulty (see below) but EF has proven to be poorly immunogenic, perhaps because it is not recognized as a foreign protein. After several efforts, reactive antisera were obtained from one rabbit and from one of six immunized guinea pigs. These sera had good titers in ELISA but did not yield precipitan bands in gel diffusion, suggesting that a single antigenic site was recognized. It is intended that these sera be tested for their ability either to neutralize the enzyme activity of brain cyclase or to immuno-precipitate specific proteins from detergent extracts of brain membranes. To date, only the "Western blot" technology has been tried. Brain extracts were electrophoresed on SDS gel slabs, the proteins electrophoretically transferred ("blotted") onto nitrocellulose sheet, and proteins recognized by the anti-EF sera were located with a peroxidase conjugate of staph protein A. Approximately 4 bands were tentatively identified as brain cyclase by this procedure. Since this method is subject to several types of artifact, it will be necessary to use additional techniques before it can be claimed that EF is antigenically similar to brain cyclase.

Further studies have been conducted on the action of EF (with PA) on cultured cells. Elevated cAMP levels slow the growth of most types of cells and are lethal to a few types, of which the S49 mouse myeloma is the best characterized. Mutant S49 cells resistant to cholera toxin or to dibutyryl-cAMP have been isolated previously and have helped to identify components of the hormone responsive adenylate cyclase system. In collaboration with Dr. Henry Bourne, University of California, San Francisco, the feasibility of isolating S49 mutants resistant to EF (and PA) is being examined. Wild type and mutant S49 cells were incubated with various combinations of EF, PA, and phosphodiesterase inhibitors (to block cAMP hydrolysis). Although growth of the cells was slowed, there was not a high degree of mortality, as would be required for mutant selection. Thus, EF may only be useful for mutant selection in S49 when combined with other agents that elevate cAMP concentrations.

Other cell lines had previously been found to survive treatment with EF and PA. These tests had employed confluent monolayers. In a more sensitive test of the inhibitory action of EF and PA, cells were plated at very low densities, toxin was added one day later, and colonies were strained after 7-10 days. Growth of nearly all the cell lines tested was inhibited by EF at 1-10 ng/ml (with PA at 100 ng/ml). Vero cells showed a unique response, being inhibited by low but not by high concentrations of EF.

Many aspects of anthrax toxin research require that sensitive and specific assays be available for each toxin component. Using the purified components, specific antisera were raised. High titer anti-PA sera were produced in both rabbits and goats. LF induced good sera in several rabbits but appeared poorly antigenic in goats. (Anti-EF sera were discussed earlier.) Though the total number of animals immunized is not large, there is a suggestion that the more highly purified PA and LF samples were less effective immunogens. This might serve to confirm an old claim that EF can act as an adjuvant, as has been established for another cAMP elevating agent, cholera toxin. Goat and rabbit anti-PA antibodies were purified on an affinity resin of PA attached to CNBr-activated Sepharose. The purified goat antibody has been used in an antibody capture type microtiter ELISA which is capable of detecting 1 ng PA. This ELISA system has been used to screen various B. anthracis strains. Sterne strains cured of plasmid (Drs. Mikesell, Knudsen) failed

to make PA (<0.1% of level produced by parent strain). This detection system has been extended to use on flat polyvinyl sheets, which is the mode needed for screening of E. coli or B. anthracis colonies for PA synthesis.

ELISA methods have also been developed for detection of antibodies to PA, LF, and EF. Though this technique was initially set up to screen for mouse monoclonal antibodies, a simple modification allows quantitation of rabbit, human, or guinea pig IgG antibodies. Antigen is bound to the plastic surface of microtiter wells, unknown or standard antisera are added, and bound IgG is detected with a peroxidase-staph protein A conjugate.

The identification of EF as an adenylate cyclase provides a very sensitive method for detection of this protein. When the Sterne strain was grown in medium supplemented with horse serum it was possible to measure calmodulin-dependent adenylate cyclase activity in less that 10 µl of culture supernate. When supernatants of parent and plasmid-cured strains were concentrated by ammonium sulfate precipitation, it was found that the Sterne cured derivative retained about 1% of the parental cyclase activity. The large decrease in toxicity associated with plasmid loss (results from Bacteriology Division investigators) had suggested that the genes for PA or LF were carried on the plasmid. The retention of some EF synthesis in the cured derivative indicates that at least one copy of the EF gene is not on the plasmid.

Attempts continued during this period to determine the mechanism of action of LF. Cultured cells treated with LF and PA were examined for alterations in amino acid uptake, nucleotide pool composition and phosphoprotein profile. No gross changes were found. However, some of these studies employed Chinese hamster ovary (CHO) cells which were subsequently found to be among the least sensitive cell lines (see below). The purified LF was tested for several enzymatic activities. No ability to hydrolyze or modify NAD or the common nucleoside triphosphates was detected. Some knowledge of the physiological systems altered by LF might be gained if a protective drug were identified. Older studies had survey some common drugs without identifying any that were protective with the possible exception of barbiturates (3). To test whether calmodulin or prostaglandins might be involved, rats in groups of 3 were given chlorpromazine, indomethacin, or saline prior to challenge with crude toxin (provided by Dr. Ivins, Bacteriology Division). All 9 rats died between 58 and 60 min. Therefore, at present no specific hypothesis as to the molecular mechanism of action of LF is favored.

The basic information needed to form hypotheses on the action of LF should evolve from the recent findings that LF (with PA) either kills or blocks the growth of many types of cultured cells. Surprisingly, CHO cells are one of the few types that resist the action of LF. This toxic effect is most clearly seen in assays like those described above for EF, where cells are plated at low density and challenged one day later. With particularly sensitive cells such as BHK, a simple cytotoxicity test based on the color change of media can be done in microtiter plates. This semi-quantitative assay should replace the cumbersome and expensive rat lethality assay. Also the fact that sensitive cells are now identifed will allow examination of what cellular functions are first affected. This information may lead to a rational search for the enzymatic activity presumably possessed by LF.

Work continued during this period on the selection of hybridomas producing monoclonal antibodies to anthrax toxin components. Increased experience with this technology did lead to greater success with individual aspects of the procedure, but successful completion of the entire process and isolation of hybridomas was not achieved. With the establishment of a hybridoma lab at USAMRIID (Dr. Early, Virology Division) this work has now been resumed. Dr. Early has performed a fusion and has cloned a number of hybridomas producing antibodies to LF. The lines will now be grown in ascites to obtain large amounts of antibody.

Studies designed to characterize and isolate the genes coding for anthrax toxin were begun during this period, and are being performed by Dr. Michael Vodkin, a senior level NRC Associate. DNA of B. anthracis was cleaved by the restriction nuclease Mbo I and ligated to plasmid pBR322 DNA that had been digested with BamHI. E. coli strain HB101 was transformed with the recombinant DNA and transformants carrying plasmids with inserts were identified by their resistence to ampicillin and sensitivity to tetracycline. The DNAs from randomly selected colonies were examined on agarose gels after digestion with BamHI, and were shown to be of various sizes. This shows that a "library" of B. anthracis genes was produced. A library was initially constructed from the DNA of a Sterne strain cured of plasmid. Attempts were also made to construct a "mini-library" from the DNA of the plasmid. However, this effort has been hampered because it proved very difficult to isolate the DNA of this large and fragile plasmid. Using the small mounts of plasmid DNA available, it has been demonstrated that cleavage with BamHI produces at least eight restriction fragments. Summing the sizes of the individual fragments gives a minimum size of 120 kilobases for the plasmid. More recently a library has been constructed for the total DNA of the Sterne strain. This library is expected to contain both chromosomal and plasmid genes. The several thousand colonies comprising this library are now being screened to detect ones producing anthrax toxin components.

Presentations:

1. Leppla, S. Anthrax toxin edema factor. Presented, Anthrax Research Review, WRAIR, 1 December 1981.

2. Leppla, S. Anthrax toxin. Presented, USAMRIID Staff Conference, 28 January 1982.

3. Leppla, S. Anthrax toxin edema factor: a bacterial adenylate cyclase. Presented, Gordon Conference on Microbial Toxins, Plymouth, New Hampshire,2-6 August 1982.

Publications:

1. Leppla, S. H. 1982. Anthrax toxin edema factor: A bacterial adenylate cyclase that increases cyclic AMP concentrations in eukaryotic cells. Proc. Natl. Acad. Sci. USA, 79:3162-3166.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL DD-DR&E(AR) 636
				DAOH 0292	82 10 01	
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9. LEVEL OF SUM A. WORK UNIT
82 04 06	D. CHANGE	U	U		NL	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	134
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE *(Precede with Security Classification Code)*
(U) Immunopathogenesis of Junin Virus Infection in Guinea Pigs and Humans

12. SUBJECT AREAS
003500 CLinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING ORGANIZATION	16. PERFORMANCE METHOD
82 04	85 04	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE		
a. DATE EFFECTIVE	EXPIRATION	FISCAL YEARS	a. PROFESSIONAL WORKYEARS	b. FUNDS *(In thousands)*
b. CONTRACT/GRANT NUMBER		82	1.3	98
c. TYPE	d. AMOUNT	83	3.0	151
e. KIND OF AWARD	f. CUM/TOTAL			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
a. NAME USA Medical Research Institute of Infectious Diseases	a. NAME Virology Division, USAMRIID
b. ADDRESS *(Include zip code)* Fort Detrick, MD 21701-5011	b. ADDRESS Fort Detrick, MD 21701-5011
c. NAME OF RESPONSIBLE INDIVIDUAL Barquist, R F	c. NAME OF PRINCIPAL INVESTIGATOR McKee, Jr., K T
d. TELEPHONE NUMBER *(Include area code)* 301-663-2833	d. TELEPHONE NUMBER *(Include area code)* 301-663-7244
21. GENERAL USE FIC MILITARY/CIVILIAN APPLICATION:	f. NAME OF ASSOCIATE INVESTIGATOR *(If available)* g. NAME OF ASSOCIATE INVESTIGATOR *(If available)*

22. KEYWORDS *(Precede EACH with Security Classification Code)* (U) BW Defense; (U) Military Medicine; (U) Vaccines; (U) Prophylaxis; (U) Junin Virus; (U) Laboratory Animals

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS *(Precede text of each with Security Classification Code)*

23. (U) Junin virus, cause of Argentine hemorrhagic fever is a potential BW threat. It is essential chat added information be obtained on its pathogenesis, so as to prepare a vaccine against the disease to protect U. S. Military forces. Information will also be directly applicable to other arenaviruses which are distributed world wide and which constitute a threat to our Rapid Deployment Forces.

24. (U) Examine replication of Junin virus within circulating lymphoreticular cells and the cellular and humoral responses to this infection. Monoclonal antibodies (designated XJC13) are available for these studies.

25. (U) 8204 - 8209 - Concentration and purification procedures were successfully developed for Junin virus in order to conduct sophisticated probes into viral pathogenesis. The virus was successfully concentrated by pelleting through a 15% sucrose and 15% to 40% continuous Renografin gradients. For the first-time, oligonucleotide fingerprints were obtained on purified Junin virus. Four distinct RNA species were identified. This information contributes directly to vaccine development studies since it partially defines the nature of the protein. Cebus sp. monkeys, when infected with the virus, do not manifest clinical illness. Serun neutralizing and immunofluorescent antibody determinations confirmed infection. Unfortunately, these data suggest that this primate will not be a suitable model for the disease, antigens that contribute tc immunity. Tests are being initiated in other species of monkeys.

BODY OF REPORT

Project No. 3A161101A91C: In-House Laboratory Independent Research (U)

Work Unit No. 91C-00-134: Immunopathogenesis of Junin Virus Infection in Guinea Pigs and Humans

Background

Argentine hemorrhagic fever (AHF) is an acute febrile disease with high morbidity and mortality. The etiologic agent, Junin virus, is a member of the arenavirus taxon, and as such possesses many biologic features of the group, including persistence in natural rodent hosts. The virus is closely related serologically and biologically to Machupo virus, the etiologic agent of Bolivian hemorrhagic fever (BHF), which produces a clinically similar disease.

The AHF clinical syndrome ranges from a mild, self-limited and non-specific illness to a severe hemorrhagic diathesis and death. Characteristically, malaise, anorexia, retro-orbital headache, and fever are followed by thoracic and facial flushing, petechiae, bleeding gums, and progressive neurologic dysfunction. Secondary infection and shock are often-observed complications of the disease, but the precise mechanisms leading to death are poorly understood. Mortality in d AHF and BHF approaches 15%; however, recent therapeutic successes through administration of human immune plasma to AHF patients have reduced this figure to less than 2%. Although effective in preventing death during the acute stages of illness, this approach is complicated by the risks of transfusion-borne diseases, volume overload, and a curious late-onset neurologic syndrome of obscure etiology that has been observed in 10% of treated survivors.

AHF has been extensively studied since its recognition as a distinct clinical entity early in the 1950's. However, many aspects of the disease's immunology and pathogenesis remain obscure. A variety of experimental animals have been used to study Junin virus infection, including hamsters, rats, mice, and guinea pigs. While each small animal model has proved useful in investigating certain aspects of disease pathogenesis, the necessity for a primate model to study unique features of the disease observed in man has been recognized. Recent successes by Argentine investigators in generating an AHF-like syndrome in marmosets (Callithrix jacchus) hold promise for future applications. Unfortunately, difficulties encountered with maintaining these animals in captivity, together with their small size, make extensive pathogenetic study prohibitive. We have therefore worked at USAMRIID on establishing a large primate model for the study of AHF.

The current work unit is in its infancy. Approval was granted less than 6 months ago, so the majority of effort has been focused on developing immunologic reagents, and animal modeling. Attention has shifted away from the initial thrust of the research unit due to the pressing urgency for developing a large primate model in which to study disease pathogenesis and protective efficacy of a promising new Junin virus vaccine candidate. The Principal Investigator is K.T. McKee, Jr.

Progress

There have been two primary lines of research persued to date: (a) studies of the molecular biology of Junin virus, and (b) assessment of Junin virus infection in 2 types of primates: Cebus sp. monkeys and rhesus macaques.

Molecular biology: In order to conduct sophisticated probes of Junin virus pathogenesis, it is necessary to have on hand reagents of high purity and specificity. Future plans call for investigation of Junin virus antigen-specific immunologic responses to infection studies which will require purified preparations of viral nucleocapsid and envelope proteins. As an initial step in obtaining such preparations, a technique for viral concentration and purification was devised. In conjunction with Dr. Patricia Repik (Dept. of Viral Biology, USAMRIID), a variety of different concentration and purification schemes were attempted, including: precipitation of virus with polyethylene glycol/NaCl and ammonium sulfate; rate gradient centrifugation through sucrose and K^+tartrate-glycerol solutions; and equilibrium centrifugation through combinations of continuous and discontinous sucrose, K^+ tartrate-glycerol, glycerol, and renografin gradients. Ultimately, virus propagated in Vero cells was concentrated by pelleting through a 15% sucrose cushion, then purified by sequential centrifugation on 20%/60% discontinuous sucrose and 15% to 40% continuous Renografin gradients. Virus was marked by radioisotope label (^3H, ^{35}S, or ^{32}P) and infectivity assayed coincidentally with radioactivity. At the end of the purification, infectivity titers of approximately 10^8 PFU/ml coincident with maximum radiosotype incorporation were observed in a single optically-defined band.

Nucleic acid characteristics were then examined. Virion RNA was extracted with a chloroform/phenol solution, and electrophoresed on 2.2% polyacrylamide gels. Four distinct RNA species were identified--large and small viral RNA's (approximately 31S and 25S), together with cellular 28S and 18S ribosomal RNAs. The 4 RNA species were then electrophoresed in two dimensions following RNase digestion to obtain oligonucleotide maps. These analyses confirmed the uniqueness of the 4 RNA's as distinct species. To our knowledge, this work represents the first oligonucleotide fingerprints obtained with Junin virus.

Preliminary steps were taken in identification and purification of viral proteins. Virus from discontinuous gradients was solubilized in SDS, and electrophoresed to locate protein bands. Virus-specific proteins were identified in conjunction with cellular proteins; further purification is in progress.

Junin virus in primates. Two primate species were chosen for initial experiments with Junin virus--Cebus sp. monkeys and rhesus macaques. Experience with Cebus sp. by Argentine investigators suggested that they might be useful as a model for AHF central nervous system disease. Sixteen monkeys were divided into groups of 4 (3 experimental and 1 control group). Experimental groups received intramuscular inoculations of 7×10^3 PFU Romero strain, 1×10^5 PFU strain 3551, or 2×10^5 PFU Junin vaccine candidate #1. Controls were inoculated with virus diluent. Animals were observed daily for signs and symptoms of disease and objective clinical and laboratory parameters followed biweekly for 4 weeks. Little or no clinical illness was observed throughout the experimental period. There was no significant difference in weight change between controls and animals inoculated with any virus strain. Hematologic findings showed inconsistent changes in white

blood cell, hematocrit, and platelet counts in Romero and 3551-infected animals. No virus was detected by plaque assay of serum, and no virus was recovered (plaque assay) from throat swabs. Serum neutralizing and immunofluorescent antibody determinations confirmed infection of all but control animals, with consistent appearance of immunofluorescent antibody by day 7-14 post-infection, and neutralizing antibody 7-10 days later (Fig. 2). Both immunofluorescent and neutralizing antibody titers were 4-5 fold less in Candidate 1-infected animals than in those infected with virulent strains.

Half the monkeys in each group were sacrificed between days 30 and 35 post-infection. Viral antigen was not detected by direct immunofluorescence in brain, spinal cord, lymph nodes, or spleen from any animal. Quantitative virus titrations of organs are pending. Histopathologic findings were remarkable for the presence of scattered foci of perivascular mononuclear cell infiltration with occassional glial reaction in the brain and spinal cord of animals inoculated with Romero and 3551. Lesions were reminiscent of those seen in poliomyelitis. Although occassional infiltrates were observed in Candidate 1-infected monkeys, the degree to which such changes were observed was minimal.

The remaining survivors were observed for an additional 2 months, and exhibited no clinical changes. At 90 days postinfection, they were sacrificed. Histopathology is pending.

Four rhesus macaques were than inoculated intramuscularly with 7.5×10^3 PFU/animal of Romero strain Junin virus, while 2 controls were inoculated with virus diluent. Again daily clinical observations and biweekly objective assessments were made. Two of the 4 infected monkeys demonstrated transient anorexia and mild depression during the second week post-infection. Mean weights of infected monkeys were decreased during weeks 2 and 3 postinfection when compared with controls. However, hematologic studies (WBC, hematocrit, and platelets) revealed no alterations, and virus was undetectable by plaque assay in the serum of infected animals. Neutralizing antibody responses generally paralleled those observed in Cebus monkeys. More complete data is unavailable at this time.

In addition to these two primary lines of research, preliminary work has been conducted in constructing an ELISA system for Junin antigen and antibody detection. Experiments performed to date have resulted in establishing parameters for performing the tests with regard to reagent concentrations and sensitivity of detection. Further work is currently ongoing. Serum specimens obtained from the above-mentioned primate experiments will be quite useful in testing these systems upon their completion.

Publications/Presentations:

None.

APPENDIX A
VOLUNTEER STUDIES

Addendum to Protocol 79-5, M-16, Evaluation of the Human Response to Administration of Rift Valley Fever Vaccine, Inactivated, Dried TSI-GSD-200 (Ind 365), FY 82-1, M-34 Addendum to M-16 (4 MRVS)

The protocol was initiated to determine the safety and immunogenicity of lots 9 through 20 of RVF vaccine, dried TSI-GSD-200. Presently, five volunteer sujects have been inoculated. None have exhibited local or systemic reactions to the initial series of three injections. One subject was dropped from the study after the second vaccine inoculation after exhibiting an anxiety reaction. Serologic results are being calculated. More subjects will be vaccinated to complete the protocol.

Immunization with Live Attenuated Dengue Type 4 Virus Vaccine, Study 1: Safety and Immunogenicity of Dengue-4 (H241) PDK 35-TD 3 FRHL-3 Vaccine (Ind 1669, FY 82-2, M-37) (3 MRVS 3 volunteers)

Five volunteers received undiluted DEN-4 (H-241/PDK 35) vaccine Lot #1 in March or May 1982. Each volunteer had no dengue antibody but was immune to yellow fever. Only two recipients seroconverted following immunization, for an infection rate of 40%. Both infected volunteers developed viremia and mild dengue-like symtoms including a macular rash. Virus isolates recovered from the volunteers retained their temperature sensitivity but produced larger plaques in cell culture than the vaccine strain, suggesting some phenotypic change had occurred.

These findings suggest the H-241/PDK-35 vaccine virus is insufficiently infectious for humans, yet still capable of producing symptomatic infections. The consensus of the investigators is that additional human testing of this vaccine is not warranted at this time.

Evaluation of WR 180,409 in the Treatment of Multi-drug Resistant *P. falciparum* (Ind 12735), FY 82-3, M-35 (7 MRVS)

WR 180,409 was administered orally to 22 nonimmune subjects infected with the multi-drug resistant Vietnam Smith isolate of *P. falciparum*. The drug was curative in single day treatment regimens down to a total dose level of 750 mg. It was not effective in one subject who was treated with a single dose of 500 mg. The drug produced a rapid clearance of parasitemia in every case and was well tolerated.

Addendum to Protocol 81-2 M-25, Evaluation of the Human Response to the Administration of Botulinum Toxoid Adsorbed Monovalent & MDPH Lot #7007 Ind 161, FY 82-4 NO-38A (8 MRVS)

Eight individuals previously given botulinum toxoid, adsorbed monovalent (E), Lot #7007, at 0, 2 and 12 weeks were given a booster injection of the same toxoid twelve months after the start of the immunization series. No local or systemic reactions were observed in any of the subjects. Serum for serology was obtained prior to booster immunization and 14, 28, and 56 days post-immunization. Titers of most subjects prior to booster immunization were lower than the previous titers. All subjects had an increased titer 14 days after the booster injection and titers were sustained through the sampling period. These data are similar to those in the literature and it appears that a complete series, i.e., immunization at 0, 2, 12 weeks and 12 month booster, is required to produce sustained immunity with this toxoid.

Safety trial Typhoid Shigella Sonnei vaccine (Strain 5076-1C) for a volunteer study at USAMRIID and WRAIR (Ind 1737) 1305-01

Progress Report

The Typhoid Shigella sonnei live oral attenuated vaccine 5076-1C is safe and well tolerated. One volunteer, who received a dose one log higher (1×10^{10} organisms) than planned for future challenge studies and field trials, developed mild abdominal discomfort, and had one watery stool from which the vaccine strain was isolated. Three other volunteers passed the vaccine strain in normal stool of the same day that they received the vaccine. Thus, the vaccine strain has a short transit time for passing through the gut in some individuals. As in previous studies with S. typhi 21a, none of the volunteers became asymptomatic carriers of the vaccine strain.

The transfer of the Form I antigen to resident gut flora was not detected in the 690 lactose positive cultures tested. None of the negative colonies isolated from the volunteers were Shigella or Salmonella species.

Only two of the ten volunteers tested developed an antibody response to the LPS of the vaccine strain 5076-1C, and one of these volunteers also developed a response to LPS from a wild strain of S. typhi. The latter response probably represents an anamnestic response since the volunteer had been immunized with S. typhi LPS previously. A serum antibody response to the vaccine strain LPS was not aniticipated since serum antibody response during natural disease with Shigella species is unusual and the serum antibodies response was minimal in previous studies with typhoid 21a vaccine.

The results of this phase I study are very encouraging. The efficacy of this vaccine will be determined in future challenge studies and field trials.

APPENDIX B

PUBLICATIONS OF U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FISCAL YEAR 1982

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APPENDIX C

CONTRACTS, GRANTS, MIPRs, AND PURCHASE ORDER IN EFFECT

FISCAL YEAR 1982

NO.	TITLE, INVESTIGATOR, INSTITUTION
DAMD-17-80-C-0154	Mechanisms of Protective Immunogenicity of Microbial Vaccines of Military Medical Significance. M. S. Ascher, University of California College of Medicine
DAMD-17-74-C-4128	Genetics of the Encephalitic vector, Culex tarsalis, for Possible Application in Integrated Control. M. Asman, University of California Berkeley
DAMD-17-78-C-8017	Genetics and Molecular Studies of the Phlebotomus Fever Group of Viruses. D. L. Bishop, University of Alabama
DAMD-17-82-C-2179	Toxicologic and Analytical Studies with T-2 and Related Trichothecene Mycotoxins. W. B. Buck, University of Illinois.
DAMD-17-82-C-2021	Studies on Immunochemical Techniques for Detection of Selected Fungal and Dinoflagellate Toxins. F. S. Chu, University of Wisconsin
DAMD-17-82-C-1027	Togavirus-specific Immune Effector Mechanisms. G. A. Cole, University of Maryland
DAMD-17-82-C-2119	Research and Preparation of an Equine Heptavalent Botulinal Antitoxin. R. M. Condie, University of Minnesota
DAMD-17-80-C-0100	Study of toxic and Antigenic Structures of Botulinum Neurotoxins. B. R. DasGupta, University of Wisconsin
DAMD-17-81-C-1028	Rapid Methods for the Laboratory Identification of Pathogenic Microorganisms. R. J. Doyle, University of Louisville
DAMD-17-79-C-9024	Lassa Immune Fever Plasma. J. D. Frame, Columbia University
DAMD-17-79-G-9494	Isolation of the Etiologic Agent of Scandinavian Epidemic (Endemic) Nephropathy from Human Patients (and from Wild Rodents) as Presumptive Strain in a Vaccine against Korean Hemorrhagic Fever. G. Friman, Uppsala University Hospital, Uppsala, Sweden
DAMD-17-77-C-7043	Department of Psoralen Photoinactivated Alphavirus and Arenavirus Vaccines. C. V. Hanson, California Department of Health
DAMD-17-77-C-7018	Vector Competence of Mosquitoes for Arboviruses. J. L. Hardy, University of California, Berkeley

MIPR-2505	Preparation and Characterization of Mouse and Human Monoclonal Antibodies to Botulinum Toxins. K. W. Hunter, Uniformed Services University of the Health Sciences
DAMD-17-80-C-0091	In Vitro Selection of an Attenuated Variant of Sindbis Virus: Investigation of the Molecular Basis for Attenuation. R. E. Johnston, North Carolina State University
DAMD-17-82-C-2188	Ion Channels from Mammalian Brain and Heart Incorporated into Planar Lipid Bilayers: Regulation by Membrane Potential, Calcium and Neurotoxins. B. K. Krueger, University of Maryland
DAMD-17-79-G-9501	Korean Hemorrhagic Fever. H. W. Lee, Korea University Medical College, Seoul
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GLOSSARY

ADCC	Antibody Dependent Cell-Mediated Cytotoxicity
AG	Antigens
AHF	Argentine hemorrhagic Fever
BAP	Bacterial Alkaline Phosphatase
BHF	Bolivian Hemorrhagic Fever
BR	Barrier-Reared
CF	Complement Fixation
CHIK	Chikungunya Virus
CHO	Chinese Hamster Ovary
CI	Chemical Ionization
CL	Chemiluminescent
CPK	Creatine Phosphokinase
CRD	Chronic Respiratory Disease
DAS	Diacetoxyscirpenol
DEN	Dengue Virus
ECD	Electron Capture Detection
ED_{50}	Median Effective Dose
EEE	Eastern Equine Encephalitis
EF	Edema Factor
EHFV	Ebola Hemorrhagic Fever Virus
ELISA	Enzyme-linked immunosorbent assay
EMS	Ethyl Methane Sulfonate
FFU	Fluorescent Focus-Forming Units
GLC	Gas Liquid Chromatography
GMP	Guanosine Monophosphate

GOT	Glutamic-oxalacetic Transaminase
GP	Guinea Pig
HTNV	Hantaan Virus
ID	Intradermal(ly)
ID_{50}	Median Infective Dose
IF	Interferon
IF	Immunofluorescent
IFA	Immunofluorescent Antibody
IHA	Indirect Hemagglutination Assay
IM	Intramuscular(ly)
IP	Intraperitoneal
IT	Intrathoracic
IU	International Units
IV	Intravenous(ly)
JV	Junin Virus
KHF	Korean Hemorrhagic Fever
LCM	Lymphocytic Choriomeningitis
LDH	Lactic Dehydrogenase
LD_{50}	Median Lethal Dose
LF	Lethal Factor
MA	Microagglutination
MBR	Monobutyrl Ribavirin
MDPH	Michigan Department of Public Health
MGF	Macrophage Growth Factor
NGF	Nerve Growth Factor
NTN	Neurotoxins

PA	Protective Antigen
PAGE	Polyacrylamide Gel Electrophoresis
PEG	Polyethylene Glycol
PFU	Plaque-Forming Units
PI	Post-inoculation
PIC	Pichinde
PMN	Polymorphonuclear Leukocytes
RIA	Radioimmune Assay
Rb	Rabbit
RLA	Radio-Labelled Antibody
RVF	Rift Valley Fever
SC	Subcutaneous
SEB	Staphylococcal Enterotoxin B
SEC	Staphylococcal Enterotoxin C
SPRIA	Solid Phase Radioimmune Assay
TLC	Thin Layer Chromatography
TCA	Trichoroacetic Acid
WRAIR	Walter Reed Army Institute of Research

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